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Neue Apparatur zur Herstellung von Schüttelkulturen von Mikroorganismen

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Summary. A simple apparatus for preparing shake cultures of microorganisms on a laboratory scale is described. Using a series of special shaking vessels, each of a capacity of 1–10 l., a total volume of 20–200 l. of culture can be produced in one run. The rate of aeration is similar to the one observed in shake flasks.

Für das Studium mikrobiologischer Verfahren im Laboratorium ist es oft notwendig, Mikroorganismen in grösserem Massstab zu züchten. Zu diesem Zweck werden meist konventionelle Schüttelkolben oder kleine Fermenter verwendet. Trotzdem diese Methoden in vielen Fällen sehr zweckmässig sind, haben sie aber auch Nachteile. Bei Schüttelkolben ohne Schikanen ist die Belüftung relativ schlecht und zudem kann ihr Effektivvolumen zu höchstens 20% ausgenützt werden.[1] Die Fermenter zeigen zwar diese Nachteile nicht, sie sind aber apparativ anspruchsvoll und stehen nicht überall zur Verfügung. Im folgenden wird eine einfache und ökonomische Methode zur Herstellung von grösseren Mengen mikrobiologischer Kulturen beschrieben, die sich in unsern Laboratorien für bestimmte Zwecke gut bewährt hat.

Bei dieser Methode handelt es sich um eine Erweiterung des Schüttelkolbenprinzips. Es werden spezielle Gefässe von 2–20 Liter Effektivvolumen auf einem einfachen Apparat geschüttelt, wobei die Innenluft laufend erneuert wird. Die erzielbaren Belüftungswerte sind dabei vergleichbar mit denjenigen, die in kräftig geschüttelten Kolben erreicht werden, und die Methode hat den Vorteil der einfachen Handhabung und der besseren Ausnützung des Effektivvolumens.

Das Schüttelgefäss

Das Schüttelgefäss, wie es in Abb. 1 abgebildet ist, besteht aus einem Glaszylinder, der an beiden Enden halbkugelig zuge-

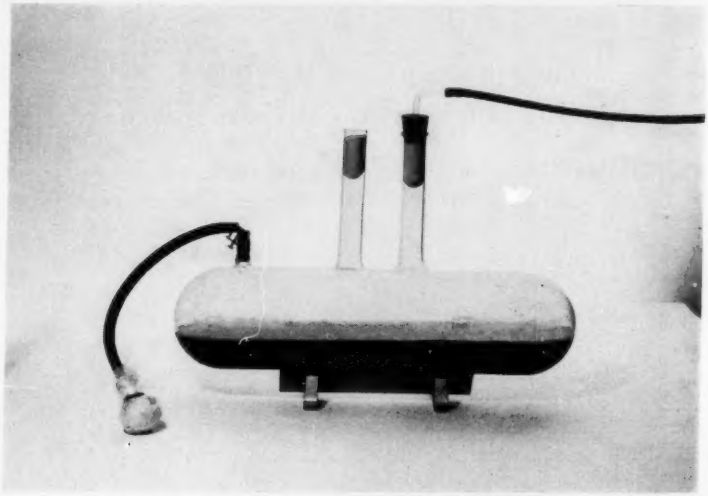

Abb. 1. Schüttelgefäss mit Kultur, ausgerüstet mit Luftzuleitung, Wattestopfen, Schlauchverbindung für Impfzwecke

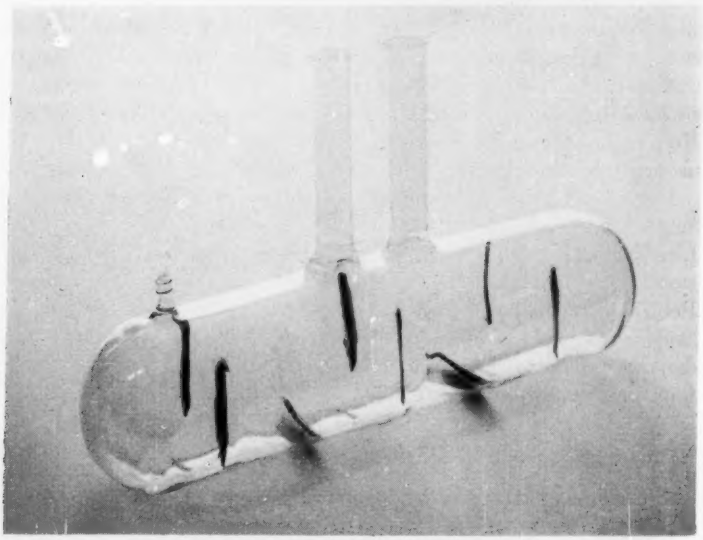

Abb. 2. Schüttelgefäss mit Schikanen

schmolzen ist. Zwei längere Rohrstutzen, am oberen Teil angesetzt, gestatten das Einfüllen und den sterilen Abschluss gegen die Aussenwelt mit Wattepfropfen. Letztere sind dank ihrer Lage vor Spritzern geschützt, die durch das Schütteln auftreten können. Durch den einen Stutzen kann frische, vorfiltrierte Luft eingeleitet werden, wobei der Wattepfropfen einen zusätzlichen Luftfilter darstellt. Der andere Stutzen dient zur Ableitung der Luft. Auf der einen Seite ist oben ein Nippel angeschmolzen, der Verwendung findet, wenn die Schüttelkultur unter sterilen Bedingungen, z.B. als Impfmaterial, in ein anderes Gefäss gebracht werden soll. Zu diesem Zweck kann, wie aus der Abbildung ersichtlich, ein Schlauch angebracht werden, der eine zweckdienliche Impfverbindung trägt.

Die Abb. 2 zeigt ein analoges, leeres Gefäss, das aber mit Schikanen versehen ist (3 Paare seitliche, vertikale, alternierend

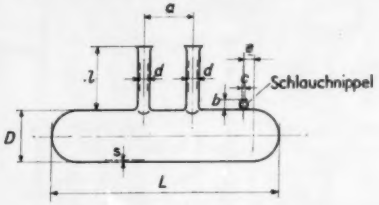

Abb. 3. Schüttelgefäss, Aufriss

mit 2 horizontalen am Boden, alle 2 cm tief und so schmal wie möglich). Diese erhöhen beim Schütteln die Turbulenz wesentlich, was vor allem bei dicht gewachsenen Kulturen wichtig ist.

Der geschilderte Gefässtypus kann in verschiedenen Dimensionen ausgeführt werden. Bei uns werden solche für 1, 3 und 9 l Nutzinhalt verwendet. Die genaue Dimensionierung ist in Abb. 3 und Tabelle I angegeben.

Tabelle I. Dimensionen der Schüttelgefässe (in mm)

Effektivvolumen, l.	Nutzvolumen, l.	$D\ \phi$ ±1 mm	L ±1 mm	s	d ϕ	l	a	b	c ϕ	e
2·5	1·0	100	400	3	33	200	85	40	8	40
10·3	3·0	160	640	3	35	225	85	40	8	40
21·8	9·0	200	800	3	35	250	85	40	8	40

Der Schüttelapparat

Der Schüttelapparat übermittelt dem Gefäss eine Hin- und Herbewegung entlang eines Kreissegmentes. Jedes Gefäss ruht auf einem Sitz und ist mit einer Klammer befestigt. Der Sitz selbst ist an einem Stab fixiert, welcher ihn mit der vertikalen Hauptachse verbindet. Die Länge dieses Stabes ergibt den Rotationsradius. In unseren Laboratorien haben sich Schüttelaggregate für 14–20 Gefässe bewährt. Die Abbildung 4 zeigt

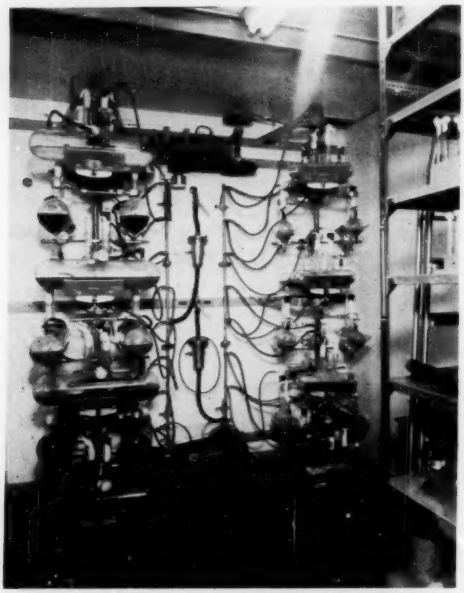

Abb. 4. Schütteleinrichtungen. Maschine links von unten angetrieben, Schüttelgefässe mit 3 l. Inhalt; Maschine rechts Antrieb von oben, Schüttelgefässe mit 1 l. Inhalt. In der Mitte Luftverteiler mit Filter

zwei solcher Maschinen und Abbildung 5 eine Planskizze. Die vertikale Hauptachse ist noch mit einer Feder verbunden, um den Motor zu entlasten und um eine einwandfreie, rucklose Hin- und Herbewegung zu gestatten. Für den Antrieb der Aggregate

mit 14 Einheiten wird ein Elektromotor von 0·5 PS verwendet. Die Luft geht durch einen Hauptfilter, der mit Watte satt gestopft und sterilisierbar ist, in ein Verteilerrohr mit 14–20 Zapfstellen. Von dort wird sie mittels Schlauchverbindungen auf die einzelnen Gefässe geleitet. Es ist zu achten, dass die Gummischläuche so gezogen werden, dass keine Reibungsstellen auftreten.

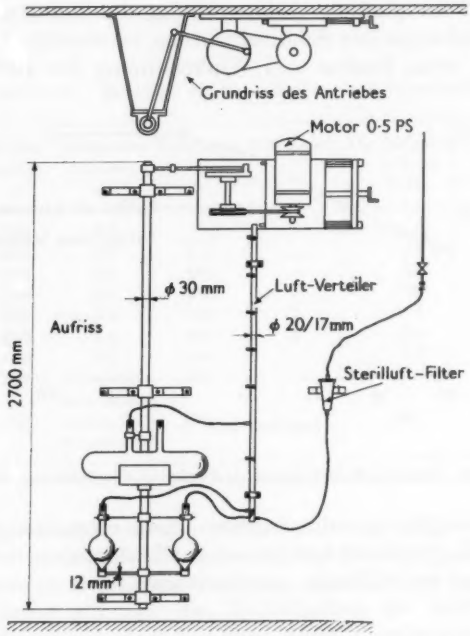

Abb. 5. Schütteleinrichtung, Grundriss und Aufriss

Die Arbeitsweise

Die Schüttelgefässe werden mit der entsprechenden Menge Nährlösung gefüllt, im Dampfautoklaven während 40 Min. bei 120°C und 1 atü sterilisiert. Zum Animpfen werden mit einer

Pipette 5–10 cc einer Sporen-, Mycel- oder Bakteriensuspension durch den einen der beiden Rohrstutzen, nach üblichem, sterilem Entfernen des Wattepfropfens, in die Nährlösung gebracht. Ueber den analogen Weg oder über Nippel und Schlauch können im Laufe der Züchtung sterile Proben entnommen werden. Die Sauerstoffversorgung der Kulturen hängt von der Intensität der Luftverteilung durch das Schütteln ab, d.h. von der Feinheit der Bläschenbildung. Zu diesem Zweck ist es notwendig, denjenigen Schüttelfrequenzbereich einzustellen, der einen kräftigen Ueberschlag der Flüssigkeit an beiden Enden des Gefässes beim jeweiligen Umkehren der Schüttelrichtung verursacht. Weiter ist es günstig, wenn Radius und Winkelöffnung der Bewegung so

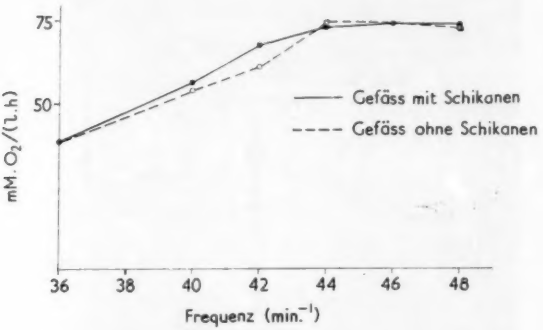

Abb. 6. Sauerstoff-Aufnahme als Funktion der Schüttelfrequenz

gross wie möglich gewählt werden. Eine zusätzliche Steigerung der Belüftung, speziell bei schweren Nährlösungen, wird bei der Verwendung von Gefässen mit Schikanen (Abb. 2) erzielt.

Die Belüftung

Mittels der Sulfit-Sulfat-Methode[2] wurde in Modellversuchen die Sauerstoffaufnahme unter verschiedenen Schüttelbedingungen gemessen. Dabei ergab sich, dass die Frequenz und speziell die Amplitude die Intensität der O_2-Aufnahme stark beeinflussen, dass aber die Menge der durch die Gefässe strömenden Luft kaum von Bedeutung ist. Die bei unserer Apparatur ermittelten

optimalen Bedingungen bezüglich Amplitude waren die folgenden: Abstand Schwerpunkt der Schüttelgefässe von der vertikalen Hauptachse der Apparatur 40·5 cm, Winkel der Hin- und Herbewegung 50°. Wie aus Abb. 6 ersichtlich ist, erreicht unter diesen Bedingungen die O_2-Aufnahme mit 75 mMol O_2/l. h bei der Schüttelfrequenz von 44 pro Minute ein Maximum, und zwar unabhängig davon, ob Gefässe mit oder ohne Schikanen verwendet werden.

In Tabelle II sind einige Werte bezüglich O_2-Aufnahme in rotierenden Kolben zusammengestellt, wie sie in der Literatur zu finden sind.

Tabelle II. Beispiele der O_2-Aufnahme in Schüttelkolben

Kolben-vol., cc	Kultur-menge, cc	Rotations-durchm., cm	Frequenz, Min. $^{-1}$	Schikanen	O_2-Aufnahme, mMol/l. h Kl_a	Autoren
500	100	5·7	0	—	6	Smith,
500	100	5·7	250	—	16	Johnson[3]
500	100	5·0	285	—	29	Chain,
500	100	5·0	180	1	190	Gualandi[1]
500	200	5·0	180	1	104	
500	200	5·0	180	2	130	
500	100	5·7	200	—	12	Corman[4]
500	100	spezielle Anordnung	220	—	16·5	Hall[5a]
500	100	spezielle Anordnung	220	1	320	

[a] Die Werte dieses Autors sind in ml O_2/(100 ml) (h) angegeben und wurden umgerechnet.

Daraus ergibt sich, dass unsere Schüttelapparatur bezüglich Belüftung eine Mittelstellung zwischen rotierenden Kolben mit und solchen ohne Schikanen einnimmt. Der Einfluss von Schaumbekämpfungsmitteln auf die O_2-Aufnahme ist merkbar. Als Beispiel sei erwähnt, dass dieselben durch Zugabe von 3‰ Sojaoel oder Silikonemulsion (D.C. Antifoam A-Emulsion der Dow Corning Co.) zur Sulfitlösung um rund 1/3 herabgesetzt wird.

Die Einsatzmöglichkeiten

Mit dieser Apparatur können die meisten, gut wachsenden aeroben Mikroorganismen kultiviert werden, sofern sie nicht einen

extrem hohen Sauerstoffbedarf aufweisen. Es wurden mit Erfolg Bakterien, Streptomyceten und Schimmelpilze gezüchtet, wobei die Kulturen zur Isolierung von Stoffwechselprodukten, zur enzymatischen Umwandlung von Steroiden oder als Impfmaterial für weitere Züchtungen Verwendung fanden. In gewissen Fällen kann diese Apparatur die teuren und in der Bedienung anspruchsvollen Kleinfermenter ersetzen.

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Production of Concentrated Cell Suspensions: the Sponge Fermentor

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Summary. A novel fermentor is described in which aerobic micro-organisms are grown on solid medium. Cellulose sponges support a nutrient-agar medium, resulting in a large surface area for growth within a relatively small volume. The sponges are alternately squeezed and relaxed within a piston-and-cylinder-type apparatus during fermentation. Provision is made for inoculation, harvesting, and for supplying air during operation. The apparatus has been operated semicontinuously in that there are enough cells left in the sponges after harvest to inoculate fresh medium. Cell concentrations of *Serratia marcescens* up to 84×10^{10} per ml have been recovered. A growth curve for this micro-organism is given and sample data on semicontinuous operation are presented.

Most commercial fermentations are now conducted in deep vat fermentors, with the prime objective of the operations being the recovery of the soluble products that micro-organisms liberate into the medium. Interest in the cell itself is usually limited to its influence on the production of the desired material. However, some exceptions to this generalization are found: where the micro-organism is of interest as a food, such as yeast or algae, where bacterial cultures are required for seed inoculation, or where the antigenic properties of certain micro-organisms are to be utilized in the manufacture of vaccines.

In the examples just cited, a considerable number of processes have been developed for the production of micro-organisms on solid medium. Some products which are now produced exclusively by submerged fermentation were originally developed on surface cultures.

Other than the mention of solid-medium growth as a laboratory technique, there is very little in the literature dealing with the engineering problems encountered in the surface culture of micro-organisms. Reed and McKercher[10] described the growth of micro-organisms on Cellophane sheets which were placed over trays

of solid medium. Concentrated cell suspensions of the order of 10^{12} cells per millilitre were reported to be harvested with a minimum of contamination by the medium. However, the yield per unit of fermentor volume was not very large.

Tyrrell, MacDonald, and Gerhardt[11] obtained populations of up to 6×10^{11} viable bacterial cells per millilitre with a biphasic growth system which consisted of a layer of solid medium overlaid with a small volume of broth of the same composition as the solid substrate. Growth was carried out in 250-ml Erlenmeyer flasks on a rotary shaker.

Despite the scarcity of engineering-type publications dealing with the surface culture of cells, many patents have been issued on mechanical devices and processes involving solid-medium-growth principles. Among the many substrates and medium supports that have been cited in patent specifications are potato tuber, dough, silica-gel, vegetable granules, oat hulls, corn bran, wheat bran, and bone meal.[2,3,4,6,7,8] The growth on one surface of a porous microfilter membrane which has its other surface in contact with a pad of absorbent material impregnated with nutrient medium is described by Goetz.[5] A process patented by Pruss and Blunk[9] describes the deodorizing of offensive gases by passing them up through a packed tower over which medium is trickling and upon which bacteria grow. Many other patents which incorporate solid-medium-growth principles have been issued for the manufacture of antibiotics, dextrose, propionic acid, and alcohol, and for the treatment of sewage and other refuse.

Solid-medium production may have certain advantages over submerged culture. The exchange of gases appears to be easier in a gas–solid system than in a gas–liquid operation. Also, a solid support medium may allow the exchange of nutrients for metabolic waste products by diffusion between the medium and the cell. If the desired product is the micro-organism, a solid-medium fermentation system might permit the harvesting of cells in a concentrated form with less contamination by the medium than would be possible by filtering or centrifuging a submerged culture. On the other hand, it seems that surface culture cannot compete economically with submerged fermentation on the basis of total number of cells produced per unit volume of fermentor. The space that would be needed in a

solid-medium fermentor for support medium could be utilized by actively growing micro-organisms in a properly aerated and agitated submerged fermentation.

In an attempt to capitalize on the advantages that might be offered by surface culture, a solid-medium fermentor was built. Cellulose sponges, of a type that is manufactured for household use, were used to support a nutrient-agar medium. The use of sponges presents the possibility of obtaining a large surface area within a relatively small volume in comparison with systems that might use Berl saddles, Raschig rings, or inert pellets to support a nutrient medium.

Sponges were used by Brown and Wood[1] for the collection of bacterial sediment for agglutinin absorption and the preparation of *Brucella antigen*. The sponges were supported in centrifuge bottles and, after fermentation using absorbed liquid medium, the broth was centrifuged out of the sponges and pipetted from the bottles.

In this experiment, the sponges were saturated with warm agar medium and the excess medium was squeezed out. The quantity of agar used in the medium was adjusted so that the thin coating of medium in the interstices of the sponges partially solidified upon cooling. The sponges were then made to absorb an inoculum of the organism to be grown. After the excess inoculum had been squeezed out of the system, the inoculated sponges were aerated by alternately squeezing and releasing them in the presence of filtered, moist air. The operation could be visualized as that of an infected human lung. At the end of the growth period of the micro-organisms, a small volume of gel diluent was added to the sponges and they were agitated violently by rapidly increasing the rate at which they were squeezed and released. The action mechanically released from the sponges a large number of organisms, which were collected as a liquid concentrate.

The apparatus used is shown in the photographs, Figs. 1 and 2. The fermentor is a one-foot length of 4-in. stainless-steel pipe that is capped on the bottom. The cap is fitted with three $\frac{1}{8}$-in. screwed needle valves which serve as inlet and outlet block valves. Within the fermentor, the sponges are supported by a heavy wire screen that was welded to a steel ring. The purpose of the screen assembly

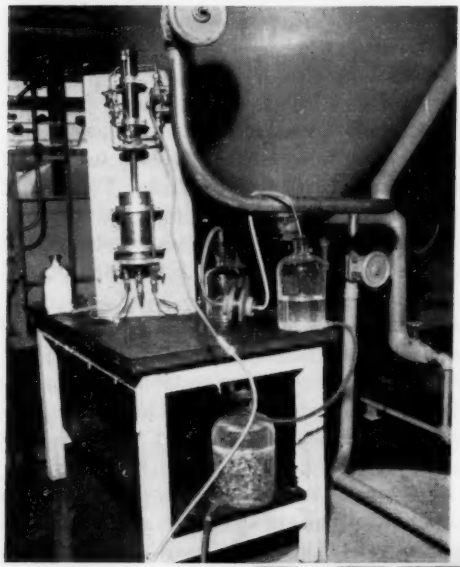

Fig. 1.
The sponge fermentor

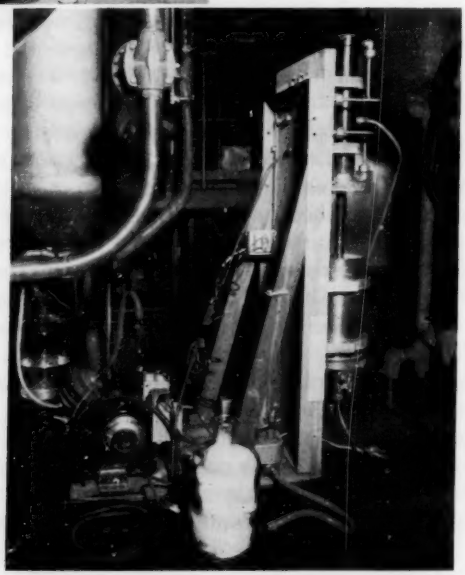

Fig. 2.
Side view of the sponge fermentor

is to provide a lower plenum for the uniform distribution of fluids introduced into the system. When the sponges are squeezed they extend through the wide openings in the screen, and touch the bottom of the fermentor, and in so doing are able to absorb any liquid that is placed in the bottom of the vessel. The sponges are squeezed by a piston which is operated by an air cylinder. A solenoid reversing switch actuates the flow of air to the motor. The flow of liquids and air to and from the fermentor is controlled by solenoid valves that are placed in line with the block valves. The operation of the solenoid control valves is synchronized with the movement of the piston by microswitches. The switches are operated by a cam that is driven by a variable speed electric motor. Normal operating speed during fluid absorption is 16 piston cycles per minute. In order to seal the fermentor at the top, a latex glove with the fingers removed is stretched over the fermentor and the bottom flange of the air motor and taped in place.

When a fermentation was started, the outlet block valve was closed and the inlet block valve opened. Warm medium was placed in a feed bottle and filtered air applied to the top of the medium. The electric motor was started and the inlet solenoid valve opened as the piston started to rise. This allowed the sponges to absorb the medium that was admitted. The inlet block valve was closed while the piston continued to operate for a period of five minutes in order to saturate the sponge. Then the outlet block valve was opened. On the down stroke of the piston, the outlet solenoid valve opened, allowing the excess medium to drain. When the medium in the system had cooled, the operation was repeated, using an inoculum of the organism to be grown. The quantity of agar used in the medium was such that, upon cooling, it would form a thin sticky paste rather than the more solid consistency that is used in Petri dishes. If the medium was too thin, most of it would be lost when the excess was drained. If it was too thick it would plug most of the openings in the sponge, preventing proper aeration, or crumble and fall to the bottom of the fermentor.

After the excess inoculum had been drained, some medium having been lost in the process, the system continued to operate with both block valves open, and sterile, moist air entering the

system. A cotton filter was used to trap aerosols of organisms leaving the fermentor with the air being discharged.

At the end of the fermentation period, diluent was admitted in the same manner as the medium and the inoculum. However, after the diluent had been absorbed by the sponges, the speed of the electric motor was increased so that the piston operated at 30 c/min in order to provide a violent agitation. The outlet block valve was opened after five minutes of washing action and the product was collected in a graduated cylinder.

Sterilization of the system was accomplished by passing a hot detergent solution through, followed by hot water rinses. The sponges were removed and washed first and then autoclaved.

Measurements were made of the volumes of medium, inoculum, diluent, and product that entered or left the fermentor. Plate counts were made on the inoculum, on the excess inoculum that was drained, and on the product.

The micro-organism used in this experiment was *Serratia marcescens*, a rather easy-to-grow, vegetative bacterium. The strain used produced a characteristic red pigment which made it easy to identify in the event of contamination by other micro-organisms becoming a problem. The medium used had the following composition: tryptose, 2 per cent; cerelose, 1 per cent; agar, 0·25 per cent; sodium phosphate monobasic, 0·02 per cent; and sodium phosphate dibasic, 0·0535 per cent. A deep vat culture of the test organism was diluted and fed to the system as inoculum or a lyophilized seed stock was reconstituted and used at an appropriate dilution. Bottles containing 99 ml of gel diluent provided the measured quantity of liquid for the harvest. Initially, when an effort was made to recover as many cells as possible by rinsing the sponges with diluent several times, two bottles of diluent were found to be a sufficient quantity to remove the majority of the cells from the sponges. The diluent was added either all at once or half a bottle at a time. Later, when concentration was the objective rather than total cells, small measured quantities of diluent were added for the harvest.

Sample data showing the concentrations of cells in the process are given in Table I. Data showing the total cells involved in the sample runs are given in Table II. The period of fermentation

Table I. Concentrations of *Serratia marcescens* in sponge fermentation

Process step	Run No.	1	2	3	4
		Cells per millilitre ($\times 10^8$)			
Fed as seed		6·33	13·20	2·40	1·10
Excess seed drained		5·23	0·25	0·43	0·04
Harvested 1st rinse		16·60	3·30	12·70	3·39
Harvested 2nd rinse		3·30	6·70	13·50	—

Table II. Total cells of *Serratia marcescens* processed in sponge fermentor

Process step	Run No.	1	2	3	4
		Total cells ($\times 10^9$)			
Fed as seed		63·3	330·0	12·0	5·5
Excess seed drained		53·4	2·1	1·6	0·3
Seed retained in sponges		9·9	327·9	10·4	5·2
Harvested 1st rinse		49·7	4·6	10·2	28·5
Harvested 2nd rinse		28·4	53·6	58·1	—
Total cells harvested		78·1	58·2	68·3	28·5
Increase over seed retained		68·2	−269·7	57·9	23·3

was 24 h for these runs. It can be seen from the data that while there was usually an increase in the concentration of cells harvested from the system, these increases cannot be correlated with the increases in total cells produced. This is partially due to the efficiency of the air humidification system. The sponges had a tendency to dry during fermentation. Consequently, they absorbed and retained different quantities of diluent when each batch was harvested. The initial moisture content of the sponges was also a factor in determining the amount of medium that was retained. Other factors may be uniformity of medium absorption, growth within the sponges, and cell removal. It is possible that high concentrations of cells might have grown in places within the sponges where they were more difficult to remove than cells growing at lower concentrations.

Attention was next directed towards running the fermentor in a semi-continuous operation, i.e. to leave the cells which could not be recovered after the harvest in the sponges as inoculum for the following run. Sample data for two runs from the

Table III. Semicontinuous fermentation of *S. marcescens* in the sponge fermentor

Process step	Run No. 1	1a	1b	2	2a	2b
1. Volume of medium fed or re-fed during operation	140 ml	50 ml	148 ml	100 ml	125 ml	125 ml
2. Concentration fed as seed	$1{\cdot}4 \times 10^8$	—	—	$19{\cdot}0 \times 10^7$	—	—
3. Total cells fed as seed[a]	$7{\cdot}0 \times 10^9$	—	—	$9{\cdot}5 \times 10^8$	—	—
4. Concentration of excess seed drained	$7{\cdot}5 \times 10^6$	—	—	$2{\cdot}8 \times 10^6$	—	—
5. Total cells drained as excess seed	$4{\cdot}5 \times 10^8$	—	—	$9{\cdot}8 \times 10^7$	—	—
6. Total cells retained in sponges	$6{\cdot}5 \times 10^9$	—	—	$8{\cdot}5 \times 10^8$	—	—
7. Volume of cells harvested	100 ml	82 ml	68 ml	62 ml	51 ml	47 ml
8. Concentration of cells harvested	$8{\cdot}8 \times 10^9$	$33{\cdot}0 \times 10^9$	$39{\cdot}8 \times 10^9$	62×10^9	69×10^9	70×10^9
9. Total cells harvested	89×10^{10}	$27{\cdot}1 \times 10^{11}$	$39{\cdot}8 \times 10^{11}$	38×10^{11}	35×10^{11}	33×10^{11}

[a] 50 ml seed suspension fed each run.

semicontinuous operation of the sponge fermentor are given in Table III. The period of fermentation was 24 h between harvests.

Although the semicontinuous series was better than the batch runs from the standpoint of cell concentration and total cell production, there was no outstanding increase in either concentration ratios or ratios of total cells between runs within a series. Since the cells were rinsed with diluent only once for each harvest during the series, it might be expected that the large inoculum retained on the sponges would result in greater increase in production than became evident.

The period of fermentation was then questioned. A possibility existed that by controlling conditions as carefully as possible a growth curve for *Serratia marcescens* in the sponge fermentor might be established by a series of batch fermentations. This was attempted by starting each run with 250 ml of medium and closing the block valve on the outlet of the fermentor when the excess medium reached a volume of 150 ml in a graduated cylinder used

to catch the excess. Fifty millilitres of a suspension of the same reconstituted lyophilized seed stock was supplied to each run. The excess suspension of seed was regulated to 35 ml in the same manner that the quantity of medium was adjusted. Plate counts made on the inoculum and drainage were fairly uniform. The concentration of the excess inoculum drained from the system averaged 47×10^5 cells per millilitre.

At the end of predetermined periods of fermentation, recovery of maximum concentrations was attempted. The sponges were violently agitated without diluent being added. By stopping the

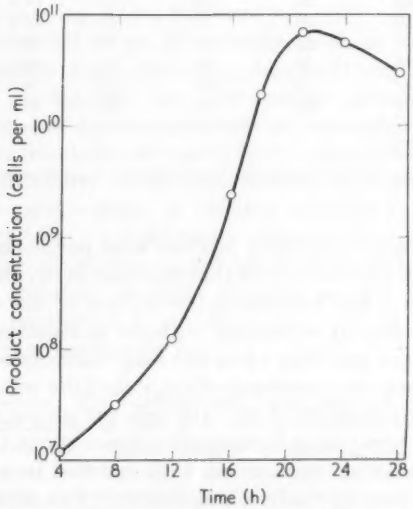

Fig. 3. Growth curve of *Serratia marcescens* in sponge fermentor

piston in the down position, pressure was kept against the sponges, allowing them to drain. It was found that for the shorter periods of fermentation the sponges were moist enough to allow the recovery of liquid product. As the length of time of fermentation was increased the sponges dried and diluent had to be added in order to recover the product. As in the case of previous fermentations, the lack of moisture control resulted in varying quantities of

product being recovered. Data from the growth curve runs are given in Table IV. These data are plotted on the graph of cell concentration *versus* time of fermentation, Fig. 3.

Table IV. Growth curve data for *Serratia marcescens* in sponge fermentor

Length of fermentation, h	Total cells seeded, $\times 10^7$	Concentration[a] of seed, $\times 10^6$ ml	Volume of diluent added, ml	Volume of product, ml	Concentration of product, per ml	Total cells recovered
4	42	28	0	42	12×10^7	50×10^8
8	48	34	0	36	49×10^7	18×10^9
12	42	28	0	33	13×10^8	43×10^9
16	30	20	0	38	38×10^9	14×10^{11}
18	47	31	20	41	27×10^{10}	11×10^{12}
21	36	24	20	48	84×10^{10}	40×10^{12}
24	32	21	25	28	74×10^{10}	21×10^{12}
28	42	28	50	31	48×10^{10}	15×10^{12}

[a] Average of 3 plates.

NOTE: All runs made using 100 ml medium retained in system and 50 ml seed suspension fed to system.

The high concentrations of product were particularly gratifying even though it was recognized that the cells were contaminated by spent medium. The average concentration of the organism produced in an aerated fermentor without mechanical agitation is 20×10^9 cells per millilitre when the same medium is used. If the deep vat fermentor is mechanically agitated the cell concentration will increase by from 60 to 80×10^9 cells per millilitre.

Replicates were run at fermentation times about the peak of the growth curve. One replicate at 18 h resulted in a product concentration of 21×10^{10} cells per millilitre. Two replicates at 21 h showed concentrations of 69 and 76×10^{10} cells per millilitre and two replicates at 24 h gave product concentrations of 61 and 59×10^{10} cells per millilitre.

When the apparatus was disassembled for cleaning, it was noticed that the bottom sponge was considerably more pigmented by the test organism than the upper sponges. This was to be expected because of the filtering action of the sponges. However, the colour was not uniformly distributed throughout any of the sponges. This may be due to non-uniform distribution of

the medium and inoculum or inadequate aeration throughout the sponges caused by a blocking of the smaller pores of the sponges with medium. Mechanically, the sponges held up very well during the experiments. The same set of three sponges were used for five or six runs. Flexible polyurethane sponges were also tried. The fermentation results were not as good and the sponges flattened out and took a permanent set after one or two runs.

Air was supplied to the fermentor at a rate of 1260 cc/min. No effort was made to control the temperature of the fermentations. These were carried out in a room in which the average temperature was about 80°F. Early runs were made in a plant where the temperatures fluctuated considerably, more particularly when equipment was being steam sterilized.

Although the full range of variables, such as the pore size of sponges, aeration rate, humidity, temperature, etc., were not investigated, it appears that the sponge fermentor might be developed for use in the recovery of concentrates of certain bacteria. The feasibility of sealing up the apparatus might depend upon the adaptability of the desired micro-organism to propagation in submerged culture or whether subsequent concentration processes might have a deleterious effect on the bacteria, as well as the mechanical problems involved in the scale-up. Even on a small scale, curiosity awakens thought on the probability of adapting the equipment to investigations such as an attempt to grow cells of mammalian tissue.

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Dihydroxyacetone: Production and Uses*

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Summary. Based on numerous reports in the literature, it appeared that the commercial production of dihydroxyacetone by microbial fermentation was not feasible due to the comparatively long duration of fermentation and the low total yield that could be obtained per fermentor. A new study has indicated that by adjusting environmental conditions and by modifying mash ingredients, commercial production of dihyroxyacetone via microbial oxidation of glycerol is practicable. The effect of these variables on the rate and extent of dihydroxyacetone production is discussed. The commercial and pharmaceutical uses of dihydroxyacetone and its derivatives is presented.

The microbial conversion of glycerol to dihydroxyacetone was first noted by Bertrand in 1898.[1] One year later, the specific micro-organism isolated by Bertrand was found to be identical with the acetic acid bacterium, *B. xylinum*.[2] Thereafter, Vissert Hooft[3] observed that aeration of the fermentation mash increased the extent of dihydroxyacetone production; 90 per cent with aeration *vs.* 57 per cent with none, after 6 days' fermentation. This was confirmed by Virtanen and Barlünd with the added observation that the optimum pH for this conversion was about 5·0.[4] Other investigators have reported that the optimum pH for the production of dihydroxyacetone was between 4·0–4·8.[5]

In connection with the glycerol concentration in the mash most favourable for dihydroxyacetone production, published reports have indicated that upon increasing the concentration of glycerine in the mash formulation, a decrease in the conversion to dihydroxyacetone was noted.[5, 6] The data shown in Table I is illustrative of the yield results obtained with increasing concentrations of glycerine in the fermentation medium. Thus,

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increasing the concentration of glycerine from 6 to 12 per cent decreased both the rate and extent of dihydroxyacetone production, i.e. 99·3 per cent yield from 6 per cent glycerine *vs.* 42·7 per cent yield from 12 per cent glycerine.

The influence of mash constituents other than glycerol on dihydroxyacetone yields has been reported by Underkofler and Fulmer.[6] Optimum yields (91 per cent after 3–7 days' aerated submerged growth) were obtained with a mash formulation containing 6 per cent glycerine, 0·5 per cent yeast extract and 0·25 per cent KH_2PO_4.

Based on the above and other reports in the literature, it was apparent that, as recently as 1947, fermentologists accepted the 'fact' that for the commercially practicable production of dihydroxyacetone by microbial oxidation, the concentration of glycerine in the mash should not exceed 6 per cent. Several years ago, our laboratory began to study this fermentation. The assay procedure employed by our laboratory for dihydroxyacetone in fermentation broths was based on a modified Folin–Wu method.[7] The potency of the white, crystalline powder final product was determined by the use of periodic acid reagent, as recommended in the official method of the Glycerine Producers Association for glycerol.[8] This procedure was not suitable when glycerol, mannitol, sorbitol and aliphatic compounds containing three or more adjacent hydroxyl groups were present.

Initial studies with various selected strains of *Acetobacter suboxydans* confirmed the observations of earlier workers, namely that with low concentrations of glycerol in the fermentation mash, high conversion yields to dihydroxyacetone were realized, whereas high concentrations of glycerol reduced the conversion efficiency. Accordingly, in order to achieve a commercially practicable fermentation, efforts were directed toward means of formulating the fermentation mash such that selected strains of *Acetobacter suboxydans* would rapidly and completely oxidize high concentrations of glycerol to dihydroxyacetone; both being necessary from a cost point of view. Complete utilization of glycerol was required since dihydroxyacetone in the presence of sizeable amounts of glycerol is crystallizable with great difficulty and the resultant crop is generally a poor product with low yields.

In connection with the above studies, it was found that the

presence of corn-steep liquor in the mash resulted in substantially complete conversion of glycerol to dihydroxyacetone in a relatively short period of time, even with mashes containing more than 10 per cent glycerol. The data in Table II, taken from shaker-flask experiments, indicate that with the addition of 0·5 per cent of corn-steep liquor to the basic 11 per cent glycerol mash resulted in a yield of 82 per cent *vs.* a yield of 44 per cent in the control mash, devoid of corn-steep liquor. On the other hand, with low, commercially uneconomical levels of glycerol, 2 per cent corn-steep liquor was without effect.

In addition to corn-steep liquor, the pH of the fermentation mash was another factor influencing fermentation yield. Thus, for example, as shown in Table III, the use of a fermentation mash with pH 5·5 afforded a yield of 82 per cent dihydroxyacetone *vs.* 29 per cent yield for the mash with a pH of 7·0.

Dihydroxyacetone has been produced in large scale fermentations and the product derived therefrom, after chemical work-up, is a white, crystalline powder with a sweet, cooling taste. This chemical has a molecular weight of 90·08 and is very soluble in water, alcohol, acetone and ether.

One of the major commercial uses of dihydroxyacetone to date is in cosmetic formulations which produce tanning of the epidermal layers of the skin, thus simulating a suntan. In this connection, dihydroxyacetone probably reacts with arginine present in the protein of the epidermis to produce the 'tanning' effect, the rate and extent of coloration being influenced by such factors as availability of free amino groups of the epidermal protein in various sections of the skin surface, the pH of the skin, the presence of inhibitors, etc.[9]

Pharmaceutically, dihydroxyacetone and its derivatives have been studied to only a minor degree. The major portion of this work was under investigation approximately 30 years ago when dihydroxyacetone was available in limited quantities. These early workers reported on the use of dihydroxyacetone in the treatment of diabetes and hypoglycaemia and its use as a respiratory stimulant.[10–16] In general, their work indicates that dihydroxyacetone when administered orally produces a less marked rise in blood sugar and a greater and more rapid rise in the respiratory quotient and oxygen consumption than glucose

Table I. The effect of glycerine concentration in the fermentation mash on yield of dihydroxyacetone

Glycerine concentration, %	Days of fermentation	Dihydroxyacetone yield, %
6	12	80·5
6	16	99·3
6	20	96·6
8	12	60·8
8	16	82·7
8	20	88·7
10	12	32·5
10	16	48·0
10	20	57·9
10	30	60·2
12	12	18·7
12	16	41·2
12	20	40·8
12	30	42·7

Data from Bernhauer, K. and K. Schon, *Hoppe-Seyl. Z.*, **177**, 107 (1928).

Table II. The effect of corn-steep liquor on production of dihydroxyacetone

Mash additives[a]		Dihydroxyacetone yield (mg/ml) Hours of fermentation	
Glycerine, mg/ml	Corn-steep liquor, mg/ml	24	72
20	0	16·5	16·2
20	5	17·8	18·0
110	0	55·0	48·5
110	5	54·5	90·0

[a] Basic mash contained 0·5 per cent KH_2PO_4, 0·5 per cent brewer's yeast, 2·0 per cent $CaCO_3$ and had a pH of 5·5–5·7.

Table III. The influence of pH of the fermentation mash on yield of dihydroxyacetone

pH of mash[a]	Dihydroxyacetone (mg/ml) Hours of fermentation		
	15	35	40
5·5	22	77	82
6·5	22	53	53
7·0	22	32	29

[a] Basic mash contained 0·5 per cent KH_2PO_4, 0·5 per cent brewer's yeast, 2 per cent $CaCO_3$, 0·5 per cent corn-steep liquor and 10 per cent glycerol in shaker flasks.

and that it was very efficient in preventing the excessive formation of beta hydroxybutyric acid and acetoacetic acid in diabetes.

Recent studies have indicated that dihydroxyacetone holds promise as an antiviral agent, its use resulting in the survival of 51–100 per cent of embryonated eggs inoculated with Newcastle-disease virus.[17]

Owing to its carbonyl group, the two primary alcohol groups, and the methylene groups, dihydroxyacetone is a very versatile molecule taking part in many reactions, including condensations and polymerizations. Thus, for example, dihydroxyacetone has been found to be the most effective reducing compound in a butadiene–styrene recipe.[18] Its use has been reported as a catalyst for the condensation of formaldehyde to hydroxyaldehydes and hydroxyketones.[19] With the availability of dihydroxyacetone in commercial quantities, numerous investigations regarding possible uses of this chemical have been and are under way.

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Continuous Cultivation of Micro-organisms: A New Approach and Possibilities for Its Use

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Summary. When trying to turn a batch fermentation process into a continuous process, many important factors must be respected. A new approach is presented by means of which it is possible to reproduce in a continuous system those changes (both inside the cells and in the environment) which are functions of elapsed time (lag-type changes, sudden changes occurring at a constant growth rate of the micro-organisms, etc.). Special attention is paid to lag-type changes. In the hitherto described apparatuses for continuous cultivation it was difficult to follow changes which are functions of time. The use of a multi-stage apparatus is therefore suggested, and a method of cultivation, which through adjusting retention times in individual cultivation vessels allows a detailed study of such changes, is proposed. Basic equations concerning the growth of micro-organisms in the described apparatus are derived and some examples of the possible practical use of the new approach are presented.

Introduction

Of the two most usual methods of cultivation of micro-organisms, i.e. the batch and the continuous methods, the latter is gaining favour as a means of solving various theoretical and practical problems. Continuous cultivation may be conducted in a single-stage or in a multi-stage system. Whereas the kinetics of batch microbial growth were studied in detail by Hinshelwood[1] and Monod,[2] kinetics of microbial growth in a continuous system were the subject of papers by Monod[3] and Novick and Szilard.[4] Málek[5] pointed out the advantages of a multi-stage system in studying some of the theoretical questions.

Very often the question to what extent it is possible to compare a microbial population growing in a continuous culture with a microbial population in a certain phase of batch growth was discussed. Since this question is of fundamental importance when changing a batch process into one of continuous-growth conditions, close attention will be paid to it in this work. This

work deals with a new viewpoint which, in our opinion, will contribute to the solution of this question. On the basis of this viewpoint, a modification of a multi-stage system of continuous cultivation is suggested. Basic equations, derived with regard to this viewpoint, characterizing the microbial growth in the proposed system are also presented.

Comparison of Batch and Continuous Cultivation

In spite of the fact that in different papers the importance of comparing both methods of cultivation was stressed, this problem was nowhere studied in a way sufficiently detailed to take into account all the important circumstances. We shall therefore concentrate our attention on some hitherto overlooked facts, the importance of which becomes apparent when considered from the viewpoint of the new approach.

Important Factors in the Comparison of Batch and Continuous Cultivations

During the growth of micro-organisms in batch cultures, changes gradually occur in the biological, morphological and chemical properties of the cells and in the composition of medium. The changes of medium composition are the result of microbial activity. To compare micro-organisms growing in a batch culture with micro-organisms growing in a continuous culture, it is advantageous to divide the properties of micro-organisms into groups according to the way in which they react to changes of the medium:

(1) Properties which change continuously with changes in the medium and are consequently a function of the actual composition of the medium (physical conditions), e.g. the concentration of ribonucleic acid in *Salmonella typhi murium*.[6]

(2) Properties which are a function of the composition of the medium (physical conditions) but not of the actual composition. They are a function of the composition of the medium as it was some time ago, or even a function of the duration of the presence of the micro-organisms in the medium; in this way they are determined by the 'history' of the micro-organisms. These

properties are consequently not only a function of the medium composition but also a function of the elapsed time.

Properties of this second type may be subdivided into two groups:

(*a*) Properties which begin to change only after a certain time after the change in the medium, i.e. lag-type changes.

(*b*) Properties which begin to change immediately after the change in the medium but do not achieve their maximum or minimum values until a certain time has elapsed, e.g. the synthesis of fats and polysaccharides in cells after thorough depletion of the nitrogen source in the medium.

It is also possible, of course, that the same property, according to the conditions, appears and disappears in a different way, and it must be emphasized that the transition from one group to another is continuous, so that many transition types exist.

The total of all the properties of the micro-organism may be called its physiological state. The evolution of the population in the culture is determined by a successive influencing of the micro-organisms in a definite physiological state by changes in the medium, irrespective of whether these changes are caused by the activity of the micro-organisms themselves or by interference from outside.

For the comparison of batch and continuous cultivations, a group of properties which undergo a lag-type change (2*a*) are important. It is therefore necessary to deal with them in a more detailed way. By lag we generally mean the time interval that passes between a certain external stimulus—a change of the medium—and the reaction of the micro-organisms to that stimulus. This time may be considered as lag only when the reaction of the micro-organisms occurs even if the medium does not change after the stimulus. That is to say, it must be ensured that the retarded reaction of the micro-organisms was not brought about by a change of environment, which attained a certain critical value and was effected by the activity of the micro-organisms responding to the original stimulus. In other words, we may consider the time interval as 'lag' only if the retardation is inherent in the micro-organism itself and is not dependent on the medium.

Concerning the lag, it is necessary to keep two circumstances in

mind. Firstly, no property of the micro-organisms can undergo a change immediately after the change in the medium. It depends on the nature of the problem how short a delay may still be considered as lag. The second fact to be kept in mind is that the retarded reaction of micro-organisms is a manifestation of a change (including appearance or disappearance) of only one particular property, which we have chosen as a rule because it will manifest itself markedly under the given conditions. This change of the chosen property may be, of course, only one single link (often the last one) of a whole chain of changes which take place in the micro-organisms during the lag. Such changes might often be observed during a detailed analysis of the micro-organisms (for instance: the known rise of sensitivity to different changes in the physical and chemical conditions during the growth-lag after inoculation, changes of the enzymatic activity during the diauxic lag, etc.). Even if these changes in no way manifest themselves in the given medium, they may have a great significance for the further development of the population in the culture, for instance because they make the micro-organisms prone to acquire a certain property which persists throughout the complete time of batch cultivation and only manifests itself in the final phase. All these changes are the effects of the same stimulus, but with lags of different duration. Some examples of stimuli for basic changes in the environment with a longer lag-time are: inoculation, entire exhaustion of a certain component of the medium and attainment of a certain critical concentration of one of the products. The lag is thus a manifestation of the necessity for deep changes in the micro-organism, which must adapt itself to a qualitatively new environment.

We shall now consider the growth and properties of a population growing in a continuous system from the viewpoint of the previously mentioned types of properties.

The Usual Method of Comparison between Batch and Continuous Cultivation and the Limits of its Applicability

It is usually assumed that micro-organisms growing in a continuous system are identical with micro-organisms in a batch culture or that they are very similar to them, assuming that the specific growth rate of the micro-organisms in both systems is

equal. When drawing a parallel between batch and continuous cultivation, the specific growth rate is usually considered to be the most important characteristic value of the micro-organisms, and in a continuous system the dilution rate is considered to be most important. This assumption is valid in many cases, because many important properties of micro-organisms are unequivocal functions of the specific growth rate. This means that the very property of the cells which determines the specific growth rate is one of a whole complex of properties, each of which depends in the same manner on the medium. Yet this point of view cannot be used if the property is not in such a relation to the growth rate but depends upon the composition of the medium in a different way. This is the case when the formation of the desired product (which is the manifestation of a certain property) is limited by a nutrient other than that nutrient which limits the formation of cell substance. For instance, during the growth of yeast, the formation of ethanol is limited by the concentration of sucrose whereas the formation of cell substance is limited by the concentration of the nitrogen source.[7]

In more complex types of media, in which either a successive exhaustion of several components of the medium or a gradual formation of a product (which may later be consumed again) occurs, probably only a few properties of the micro-organisms will be related to the growth rate in such a way. In a single-stage continuous cultivation, the micro-organisms must be equipped with enzymatic apparatus able to assimilate all the new inflowing nutrients.* In contrast, in a batch culture the enzymatic systems undergo changes in accordance with the successive utilization of the individual nutrients. In these cases, it was endeavoured to establish an analogy between the batch and the continuous processes by using several stages. In this way the different phases of batch culture (from the point of view of successive consumption of the different substrates or formation of products) are separated into several stages.[8, 9] By this method it is possible to obtain a certain analogy between the continuous

* In a continuous system, when the dilution rate values are low, all the different sources of carbon or nitrogen are utilized at the same time and the diauxic phenomenon does not occur. On the contrary when the dilution rates are higher only some of the nutrients are utilized.

process and the course of development in the batch process, but the specific growth rate as a common characteristic is not applicable. It is important to know what properties the micro-organisms have in the different stages and about this the specific growth rate can give no information.

If in the course of growth in a batch culture changes in the properties of the microbial population of the lag-type occur, it is not possible to compare the batch and the continuous processes by the hitherto usual approach. This is illustrated very well by the case described by Finn and Wilson[10] in which the specific growth rate was a 'lagged' function of pH (duration of the lag, 30 min). Monod's theory assumes that the specific growth rate is not a 'lagged' function of the composition of the medium. This circumstance resulted in this case, where Monod's assumption was not valid, in oscillations of the microbial population. No steady state was attained. Lag-type changes do not always manifest themselves in such a visible way, as was the case in the above-mentioned example in which only the concentration of micro-organisms oscillated. The reason here was that the 'lagged' function of pH was the property that determined the specific growth rate. If another property is lagged, the concentration of micro-organisms will be maintained constant, but other properties, which may even escape notice, may either oscillate or, especially when their changes do not influence the medium, change gradually so that the population becomes stabilized after a generally infinite time in a certain equilibrium state. This state, however, may differ markedly not only from that in batch culture during the time when the micro-organisms have the same growth rate as in the continuous system, but in an extreme case from any state which may ever occur in the batch culture.

Results similar to those caused by lag-type changes may also be brought about in the population by micro-organism property changes of the type 2*b*.

References to several cases exist, in which the population properties during continuous cultivation change in relation to time (cf.[11, 12, 13]).*

* We have not included those cases where the changes were provoked by genetic changes in the population. Genetic changes are not directly connected with the subject of this paper and are not considered.

On the basis of published data, it is usually impossible to decide whether properties of type *2a* or *2b* caused the observed changes, as the studies as a rule had different aims.

As to those properties which undergo lag-type changes in a one-stage continuous system, it is impossible to reach a steady state in which the micro-organisms would be identical with those in a similar state in a batch culture. A proof of this may be found in a paper by Luedeking and Piret.[14] During single-stage continuous cultivation these authors failed to obtain the growth lag in the region in which it should have appeared according to batch-growth-curve data.

Retention Time in Individual Stages—The Basis for the New Approach to Continuous Cultivation

When we try to reproduce any kind of lag-type change in a continuous system, we must use a set-up in which the population continuously overflows into the cultivation vessel under conditions which will initiate these lag-type changes. For detecting the individual phases of the lag, it is necessary to use more stages and a faster flow, so that in the period between the stimulus and the retarded reaction the population passes through several vessels. As lag-type changes in the properties of micro-organisms are a function of time, it is useless to consider the dilution rate for their reproduction in a continuous system; all-important, however, is the retention time of micro-organisms in the individual vessels. Although the retention time only represents the reciprocal value of the dilution rate, this concept should be used as a new approach which makes it easier to reproduce the lag-type changes in the continuous system. In the n-th stage, the micro-organisms are in a lag-phase which corresponds to the sum of the retention times in the individual single stages, from the stage when the lag was initiated to the n-th stage. In a similar way, not only the growth lag but other kinds of lag as well may be reproduced; it is only necessary for the micro-organisms to encounter in a certain vessel conditions which would initiate the necessary lag-type changes.

The application of this idea is not limited to the examination of certain lag-type changes only, but it is also possible to reproduce

to a remarkable degree the complete development of a population in a batch culture. At the same time all gradual changes of conditions which the micro-organisms go through during batch cultivation will be respected. With properly selected retention times in the single stages, it will also be possible to fix the times at which the micro-organisms encounter changed conditions, and how long they will remain in these conditions. This ensures that the 'history' of the population (with certain restrictions) will be, at the n-stage, the same as it is in the batch culture, at a time which equals the sum of the retention times from the first to the n-th stage. There is no guarantee of this in the usual process of comparing batch and continuous cultivations, where the retention times in the individual stages are not considered.

If, in a continuous system, we wish to get the closest possible analogy with the development in a batch culture, the use of the following arrangement of the continuous apparatus is necessary. The largest possible number of cultivation vessels is connected in a row. Fresh medium from a storage vessel is fed to the first vessel (in which the dilution rate is greater than the maximum specific growth rate of the micro-organisms in the medium) and, simultaneously, a small amount of the culture withdrawn from some of the other vessels is reintroduced into the first vessel. In this way the recycled culture is identical with the inoculum used for inoculation of the batch culture. An inflow of culture from another vessel, and not from a chemostat or turbidostat, has been used because this inoculum more closely resembles that used in batch culture, where it may be obtained by withdrawing a part of the culture from a previous batch culture. Inoculum taken from a chemostat or turbidostat might, for the above-mentioned reasons, differ after some time from an inoculum from a batch culture.

It must be remembered that (as follows from the mathematical analysis), when using fewer stages, the concentration of the micro-organisms in the n-th stage will not be exactly identical with the concentration in a batch culture at a similar time. That is why the concentrations of various medium components will not be absolutely the same. How great this difference will be and whether it will influence the development of the population depends on other factors which are outside the scope of this

article. The significance of the above-mentioned circumstance must be appraised in every single case individually.

Reproduction of the batch curve in the set-up described represents only one of its many possible uses. This set-up may be adjusted in various ways according to the scope of the work which is to be pursued, e.g. the volumes of single vessels may be changed, at the same time altering the retention times. There is also the possibility of changing the conditions in single vessels, both chemical (special feeding of nutrients, inhibitors, maintaining of pH, etc.) and physical (temperature, etc.). In this way we may follow the influence of a certain treatment in a certain phase of development on the further 'history' of the population, etc. Inoculation of the first stage from some further stage may not be necessary for some purposes, and very often the use of a chemostat or turbidostat is permissible because the different 'histories' of inoculated matter are immaterial. This subject will be treated more fully later in connection with the possibilities of the application of such a system.

As the apparatus described represents fundamentally a multi-stage continuous system with recyclization to the first stage, its differences as compared with previously described types of apparatus will be briefly summarized:

(1) The lag-type changes initiated through inoculation are not respected.

(2) Since the cultivation in the first stage usually operates at a lower dilution rate than the maximum specific growth rate over a long period, the properties of the micro-organisms may change in an uncontrolled way.

(3) When retention times are disregarded, single phases of the lag-type changes cannot be detected, unless by chance. As a rule they escape detection because the retention times in the cultivating vessels are usually long compared with the length of lag.

(4) There is no guarantee that the population will develop in the same way as that of the batch cultivation, because the micro-organisms in a certain phase of their development will not necessarily find the same conditions in the medium as they would in a batch culture at the corresponding time.

(5) As the arrangement is usually one with a small number of

large vessels, the retention times of micro-organisms in individual stages are long, producing a very inhomogeneous population, as will be shown in the mathematical part.

Mathematical Interpretation of Micro-organism Growth in the Continuous System Described from the Viewpoint of the New Approach

The basic equations for micro-organism growth in a continuous system will be derived from the viewpoint of the new approach. It will be shown in particular that as the number of stages increases the results for continuous growth approach those for batch growth. Although from the point of view of our new approach the properties of the micro-organisms are most important, in this treatment only the microbial concentration will be considered, as in all other mathematical interpretations based on specific growth rate.

For the sake of simplicity, we will proceed from the following assumptions: perfect and immediate mixing at every stage, equal volumes at all stages (and hence equal retention times), and the same specific growth rate in all the stages, equal to the maximum specific growth rate for the given micro-organism in a given medium.

Symbols used in the mathematical treatment:

x_0	Concentration of the micro-organisms in the inoculating culture entering the first vessel, g/ml
x_n	Concentration of micro-organisms in the n-th vessel, g/ml
X_n	Total quantity of micro-organisms in the n-th vessel, g
F_0	Flow rate of inoculating culture in the first stage, ml/h
F_1	Flow rate of the fresh medium in the first stage, ml/h
V	Total volume of the system, ml
m	Total number of stages
V_m	Volume of a single stage, ml
n	Number of stages considered
θ	Mean retention time for one stage, h
t	Time, h
Δt	Mean retention time for the whole system (for batch cultivation, the time elapsed since inoculation), h
D	Dilution rate, h^{-1}
k	Specific growth rate, h^{-1}

In the first stage, fresh medium is fed at a rate F_1 and inoculating culture at a rate F_0, while the concentration of micro-organ-

isms in this culture equals x_0. The equation of mass balance for the first stage will therefore be:

$$\frac{dX_1}{dt} = x_0F_0 + x_1kV_m - x_1(F_0 + F_1) \tag{1}$$

At equilibrium, $dX_1/dt = 0$, and for x_1 we get:

$$x_1 = \frac{x_0(F_0/V_m)}{[(F_0 + F_1)/V_m] - k} \tag{2}$$

By definition

$$\frac{F_0 + F_1}{V_m} = D = \frac{1}{\theta} \tag{3}$$

and substituting (3) in (2) gives

$$x_1 = \frac{x_0(F_0/V_m)}{(1/\theta) - k} \qquad \text{for } 1/\theta > k \tag{4}$$

For the calculation of microbial concentration in the n-th vessel from the concentration in the first vessel the following equation is valid (derived by Luedeking and Piret[14]):

$$x_n = \frac{x_1(1/\theta)^{n-1}}{[(1/\theta) - k]^{n-1}} \tag{5}$$

For the concentration in any vessel of the system, substituting (4) in (5), rearranging and substituting (3) gives us:

$$x_n = \frac{x_0F_0}{F_0 + F_1}\left(\frac{1/\theta}{(1/\theta) - k}\right)^n \tag{6}$$

If we now express θ as a fraction of the retention time of the whole system:

$$\theta = \Delta t/m \tag{7}$$

and substitute (7) in (6), we get as an expression for x_n:

$$x_n = \frac{x_0F_0}{F_0 + F_1}\left(\frac{m}{m - k\Delta t}\right)^n \tag{8}$$

For the concentration in the last stage, x_m, the following equation is valid:

$$x_m = \frac{x_0F_0}{F_0 + F_1}\left(\frac{m}{m - k\Delta t}\right)^m \tag{9}$$

By calculating the limit of equation (9) for $m \to \infty$ we get:

$$\lim_{m \to \infty} x_m = \frac{x_0 F_0}{F_0 + F_1} e^{k \Delta t} \tag{10}$$

As the number of stages is increased, the microbial concentration in the last stage of the system approaches the value given by equation (10). The mean retention time for the whole system and its total volume naturally remain the same, but the volumes of single stages and the retention times in them diminish. If such an idealized system with an unlimited number of stages is in operation, then, during the time Δt, amounts $F_1 \Delta t$ of fresh medium and $F_0 \Delta t$ of inoculating culture at a microbial concentration x_0 are introduced. During the same time a volume of culture equal to the total volume of the system escapes from the system, i.e. $V = (F_0 + F_1)\Delta t$; the concentration of micro-organisms in this outflowing culture is given by equation (10).

Should similar amounts of fresh medium and inoculating culture (with a microbial concentration x_0) corresponding to those entering the above system in the course of the time Δt be mixed together all at once and left to grow batchwise, the microbial concentration after time Δt would be equal to the right-hand side of equation (10).

It is therefore apparent that with increase in the number of stages, the total result of growth within a time Δt in such a system approaches exactly the result of growth of a batch culture within the same time. The initial state is the same in both cases, i.e. the same volumes of fresh medium and the same volumes of inoculating culture, with a microbial concentration x_0, are used. But whereas in the batch culture the fresh medium and inoculating culture are mixed together all at once, in the system described they are mixed together continuously. A culture volume element passes through the system within a time Δt and in single vessels single phases of its development may be observed.

The similarity of microbial growth in the continuous multistage system with that of batch growth is not surprising. The system just described represents, under given conditions, a CFSTR (continuous flow stirred-tank reactor) system, and this system, with an increasing number of stages, approaches a tubular

reactor with piston flow which is analogous to a batch reactor. For chemical processes, of which microbial growth is only a particularly complicated case, this has been derived mathematically by Denbigh.[15] The idea of our paper is based to a considerable extent on the application of the basic characteristics of a tubular reactor (i.e. space division of single time phases of batch reaction) to the development of microbial populations. Previously, all systems for continuous micro-organism cultivation have been treated as CFSTR systems. Only Herbert[17] discusses the application of a tubular reactor system with piston flow to the growth of micro-organisms. The possibility of applying a tubular reactor system to the growth of micro-organisms is seen only in the application for some practical purposes (waste-disposal problems).

Obviously, the continuous reactor system with a limited number of stages will not correspond exactly to a batch reactor one. The value x_m in equation (9) decreases with increase in the number of stages (with an infinite number of stages it would eventually drop to the value in a batch culture), so that the micro-organism concentration will be higher and the concentration of components being consumed will be less in a continuous than in a batch culture in a time equal to the sum of the retention times from the first to the n-th stage. This accords with Danckwerts' finding that CFSTR is more favourable for autocatalytic microbial growth than a batch reactor as far as accomplishing the reaction quickly is concerned.[16] From the economical point of view this circumstance has been discussed, for example, by Deindoerfer and Humphrey.[18]

It should be mentioned that the microbial concentration in the $n-1$ stage is lower than the microbial concentration in a batch culture in a time equal to the sum of the retention times from the first to the n-th stage. For this reason, the values x_n, although they are higher than the values on the batch curve, are associated with them in a quite definite way and with a greater number of stages are very close to them.

In this paper a more detailed analysis of a system with a limited number of stages or an analysis using less simple assumptions (differing volumes of the single stages, non-constant specific growth rate, etc.) is impossible. Our purpose was merely to

show the similarity between a continuous and a batch system from the point of view of retention times in single stages.

A very important question which deserves a brief mention is that of the homogeneity of the growing population in such a system. As the retention times in CFSTR are spread over a broad range around the mean retention time, only a major part of the population (and not the whole population) will have the same 'history' in the n-th stage—it will have passed through the same conditions in the medium in the same time as the population in the batch culture, in a time equal to the sum of the retention times from the first to the n-th stage. In each further stage, the population will be less homogeneous than in the previous one. In the last stage, and therefore in the whole system, it will be more homogeneous the more stages have been applied for subdividing a certain time interval of the batch process into single stages. It is impossible to deal more fully with the problem of homogeneity here.

The application of the greatest possible number of stages is advantageous therefore both for attaining the best possible analogy with the batch culture, and for obtaining a homogeneous population. For technical reasons, it is not possible to raise the number of stages above a certain limit. The most advantageous compromise solution depends on the nature of the problem examined.

Some Examples of the Application of the New Approach

The method of continuous cultivation described is analogous to that of batch cultivation. It is therefore unlikely to be of use in solving problems unsuitable for batch cultivation. In many cases, however, the new method of continuous cultivation may speed up and facilitate a solution, since the time development of the batch culture is here divided in single cultivation vessels. In addition, when compared with batch culture better reproducibility of results is ensured.

The withdrawal of samples from a batch culture in a certain phase of development is replaced by sampling from a certain stage. Instead of an intentional change in conditions at a given

moment in a batch culture, maintained for a definite period of time, we may maintain these conditions in one or more stages of a continuous culture; the volumes of these stages determine the time for which the population is exposed to the new conditions. For this reason, instead of several parallel experiments it is sufficient to perform an analysis in a certain stage at several time intervals (as a proof of the steady state). In addition, in the same run several different experiments can be performed, whereas batch culture must always be discarded.

Application of the aforementioned aspects may be necessary in certain cases for a systematic and rational conversion of a batch process to a continuous one for practical purposes when identical results are required.

Analyses of Lag-Type Changes

The simplest example of the application of the continuous system described is in the investigation of microbial properties during lag-type changes. This possibility of similar arrangements for the measurement of the length of the growth lag has been mentioned by Novick in the discussion at the symposium on continuous cultivation in Prague.[19] Two stages are proposed, the first one serving as a chemostat and fresh medium being supplied separately to the second one, where the dilution rate is higher than the maximum specific growth rate. If a lag phase occurs, its length may be calculated on the basis of the deviation of the bacterial concentration actually attained from that calculated assuming that a lag phase will not occur.

When trying to detect even the single phases of lag, however, we must, as was mentioned above, use an arrangement in which the micro-organisms will meet in some vessel, usually in the second, conditions initiating the lag-type changes; the single phases of lag will then stretch over the subsequent stages. It is obvious that the shorter the investigated lag, the shorter the retention time that must be chosen. A chemostat or a turbidostat may be used as the source of micro-organisms or micro-organisms may be obtained through recyclization from further stages, etc., according to the nature of the problem. Furthermore,

it must be established that the medium in those vessels where the lag is observed does not change and thus does not influence intracellular processes. For this reason, if the micro-organisms do multiply during the lag (i.e. if no growth lag is concerned), the application of the lowest possible microbial concentration is of advantage.

Thus, it would be possible to follow changes occurring in cells after the transfer of micro-organisms from a medium with a lower maximum growth rate to a medium with a higher maximum growth rate and vice versa. In the first stage, micro-organisms would be cultivated in one type of medium and another type of medium would be fed in surplus to the second stage. Such batch-culture experiments have been performed by Kjeldgaard, Maaløe and Schaechter.[6] They established that following the transfer of micro-organisms to a medium of higher maximum specific growth rate a dissociation of the basic synthetic activities occurs as follows: the rate of synthesis of ribonucleic acid attains the value characteristic of a medium of higher growth rate practically immediately, the synthesis of cellular material within five minutes approximately, that of desoxyribonucleic acid within twenty minutes, the number of nuclei per cell within fifty minutes approximately, and the rate of cell division in about seventy minutes.

Some other authors[20] examined the influence of a sudden change of temperature and described similar marked differences in the rate of reaction of individual synthetic activities following such a change. These experiments may also be performed in a continuous way by maintaining elevated or lowered temperatures in the second and further stages with respect to the first stage. Thus, it would even be possible to perform synchronization experiments in a continuous way.

These examples have been mentioned by way of illustration only and practice will show whether the application of a continuous system is likely to prove more favourable. Even the investigation of the influence of the time of growth under new conditions on the length and the nature of lag after returning to the previous conditions may be examined, for example, by means of a gradual recyclization from further stages into another similar continuous system.

Establishing the Causes of Some of the Changes Occurring During the Batch Process

Application of the apparatus described is by no means restricted to the examination of changes in the micro-organisms and to the detection of quite definite lags where the initiating change of the medium is well known. There are numerous cases in which changes occur during batch cultivation that cannot be directly referred to some apparent change in the medium. As a typical example we may note changes in the course of the exponential phase where a comparatively small concentration of cells influences the environment only negligibly. We shall examine only one case of this kind, which is also of practical importance. Ribosidation of azauracil proceeds most efficiently at the very beginning of the exponential phase. For this reason, Řičica[21] proposed a two-stage arrangement with an inflow of fresh medium into the second stage, which also favours the possibility of maintaining the population at the maximum growth rate without photoelectric control. In this way, a three-fold increase in azauracil riboside yield has been obtained.

Should more essential changes in the medium occur in the later phases of batch growth, things become more complicated. Thus, it cannot be decided *a priori* whether the reversion in a batch culture from the production of some product to its consumption (unless it should occur as a result of exhausting some nutrient) has been caused by its attaining a certain level in the medium or whether it would begin to be consumed even at a lower concentration. Without closer observation, therefore, a 'lagged' effect of some previous impulse may not be distinguished from a reaction caused by a coinciding change in the medium, or from a 'lagged' effect modified through changes in the medium. The solution of similar problems may be very involved and time-consuming because of the considerable length of some lags. Rogers,[12] for example, while studying the formation of hyaluronidase by *Staphylococcus aureus*, observed that the specific rate of its formation kept increasing long after the thiamine in the medium had been exhausted, which was the cause of an eventual drop in the production rate.

The continuous system may well be applied to such studies

because it permits both the composition of medium and the physiological state of the micro-organisms to be varied at the same time, more or less independently. If, for instance, it is desired that some component should be exhausted later than in a batch culture, this may easily be accomplished by adding it separately to the proper stage, independent of the main flow. Similarly, the concentration of the product may be lowered or raised by diluting the contents at a certain stage by the addition of a suitable medium not containing the product or by the addition of the product.

In this way, micro-organisms of a similar 'age' (depending upon spontaneous intracellular processes) may be placed in conditions similar to those which would prevail in a batch culture at different periods of time and so lagged changes may be differentiated from changes caused by actual changes in the medium. It is equally easy to determine the time at which the micro-organisms should find themselves in a certain condition either by prolonging or shortening the retention time in a given stage or by maintaining these conditions in a number of stages.

Study of and Influencing Population Development

The new approach may also be applied to the study of the whole population development problem and to the estimation of the influence of the 'history' of the population on the direction of its development. Pertinent questions are those of sporulation, of the appearance of the resting cells, of the development cycles and of establishing the conditions the population has to pass through in order that a given action may occur.

During batch cultivation, the sequence of changes is determined by the properties of the inoculum, its size, the type of medium and the physical conditions of cultivation. In the continuous system described one may, through a suitable arrangement, simulate at will any definite 'history' of population. By changing the volumes, temperatures, intensity of aeration, pH values and concentrations of individual medium components (nutrients, inhibitors, precursors, etc.) in the various stages, it is possible to determine influences necessary for reaching the final stage of

development of the culture, including the exact moment at which these influences must exert their action and their duration. This can be of great significance, particularly when turning the batch production process of some complicated compound into the continuous process. This applies especially in the case of compounds whose synthesis is accompanied by an increase in free energy, and is independent of either the growth of the micro-organism or the consumption of some substrate in the medium.[22]

Usually, whenever a batch process is to be reproduced in a continuous system, two stages are considered (occasionally three); in the first stage optimum conditions for growth prevail, whereas in the second and third stages optimum conditions for product formation are maintained. For optimum production, however, a different and more difficult way of simulating batch development may prove to be necessary. This requires the use of more stages for fixing the important critical points. An interesting example of the influence of a slight, very specific intervention in a definite period of development of a batch culture is the influence of interrupted aeration on the final production of chlorotetracycline by some strains of *Streptomyces aureofaciens*. It has been established that an interruption of aeration between the 6th and 10th hours for 5 min results in a dramatic drop in chlorotetracycline yield.[23] When the aeration is interrupted at some other time, even for a much longer period the undesirable effect is much less pronounced. In this particular case the production falls, but it is possible that a similar slight specific intervention at a definite time in batch culture or at a certain stage in the continuous culture might on the contrary raise the production.

In this present paper all of the questions associated with the subject have by no means been exhausted; some of them have not even been mentioned. Our aim was in the first place to call attention to some of the circumstances that may complicate the continuous cultivation and to emphasize the necessity of being more critical when comparing the population cultivated continuously with the batch population. Although the above-mentioned complications obviously do not appear in all experiments with continuous cultivation, we assume that it may be useful to consider the problem from a new and different viewpoint.

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Volume Increase of Wheat Kernels Accompanying Absorption of Liquid Water

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Summary. The purposes of this investigation were to determine the relationship between weight and volume increases which take place when wheat kernels are immersed in water, to develop an equation for the volume increase, and to test experimentally its validity.

It was shown that the volume increase was practically equal to the weight increase divided by the fluid density. In view of this, the following equation was obtained by analogy to the diffusion equation derived by Becker.[1]

$$1 - \bar{V} = \frac{2}{\sqrt{\pi}} \frac{S}{V} \sqrt{(D_v \theta)}$$

where $1 - \bar{V}$ is the relative volume increase, and D_v is the coefficient of volume increase.

The average coefficients of volume increase are given by the Arrhenius relation as:

$$D_v = 1{\cdot}068\, e^{-10{,}129/RT} \quad \text{for Ponca wheat}$$
$$D_v = 1{\cdot}240\, e^{-11{,}030/RT} \quad \text{for Seneca wheat}$$

The results obtained in this investigation provide useful information for designing the steeping vessels.

Introduction

The penetration of water into the wheat kernel is a problem of practical and theoretical importance to the wheat-milling industry.

In the past, many investigations concerning water penetration into wheat kernels have been made. These investigations, however, were mainly concerned with the rate of weight increase of the wheat kernel due to the absorption of water and with the mechanism of water penetration into the wheat kernel.[2, 5–10]

It is customary to steep the grains by circulating water through a stationary packed bed of the grains. A knowledge of the volume gain during the steeping operation, therefore, is of no less importance than that of weight gain from the standpoint of optimum sizing of steeping vessels.

Up to the present time, few works on the volume increase of wheat kernels during immersion in water have been reported. In this investigation, the weight and volume increases of wheat kernels during the steeping operation were simultaneously measured at various time intervals and temperature levels and the relationship between the weight and volume increases was investigated. An equation for the volume increase (by analogy to the weight-increase equation derived by Becker[1]) was developed, and its validity was tested experimentally.

While much information has been obtained concerning what happens in the wheat kernel during the immersion in water, there is still much groundwork left to be done. A quantitative knowledge of volume increase would certainly contribute to it.

Theory

It had been demonstrated that a Fick's law model of molecular diffusion could be used to correlate the weight increase of wheat kernels during the immersion in water.[2] Becker[1] formulated the non-stationary-state diffusion of solids for arbitrary shape from Fick's law of diffusion, assuming that the volume to surface-area ratio, V/S, and diffusion coefficient, D, are constants. The equation obtained is:

$$\bar{C} = 1-(2/\sqrt{\pi})X + BX^2 \qquad (1)$$

where $\bar{C} = (\bar{c}-c_s)/(c_0-c_s)$, in which $\bar{c}$ is the average concentration, c_0 the initial concentration, and c_s the concentration at the bounding surface; $X = S/V\sqrt{(D\theta)}$, and B is a dimensionless constant.

Since equations similar to the equation of Fick's law of molecular diffusion, which is a phenomenon of mass transport, had been applied successfully to other transport phenomena such as transport of heat and momentum under laminar flow, it might be expected that the volume change accompanying diffusion could be expressed by a similar equation, when it is considered that this volume increase is due to the transport of volume from one phase to the other phase of the material system.

Thus, equation (1) can be used directly to represent the rate of volume increase by substituting the corresponding volume term for the concentration one. Thus:

$$\bar{\bar{V}} = 1 - \frac{2}{\sqrt{\pi}} X_v + BX_v^2, \tag{2}$$

$$\bar{\bar{V}} = \frac{\bar{V} - V_s}{V_0 - V_s} \tag{3}$$

and

$$X_v = (S/V)\sqrt{(D_v\theta)} \tag{4}$$

where V_s is the effective surface volume content, V_0 the initial volume content, and D_v the coefficient of volume increase.

For small values of X_v, equation (2) approximates to:

$$1 - \bar{\bar{V}} = (2/\sqrt{\pi})\, X_v \tag{5}$$

or, in terms of experimental variables,

$$\bar{V} - V_0 = K_v\sqrt{\theta} \tag{6}$$

where $K_v = (2/\sqrt{\pi})(V_s - V_0)(S/V)\sqrt{D_v}$.

Therefore, if this equation is valid, the volume increase of wheat kernels during the immersion in water should be approximately proportional to the square root of the absorption time.

Experimental

The varieties of wheat used in this study were Ponca (hard winter wheat) and Seneca (soft wheat). Ponca wheat with an initial moisture content by volume of 22·42 per cent (dry basis) and Seneca wheat with an initial moisture content by volume of 18·42 per cent (dry basis) were used throughout the experiments. The protein contents of the Ponca and Seneca wheats were 12·7 per cent and 10·5 per cent respectively.

The effective surface volume contents of Ponca wheat and Seneca wheat were 1·04 cm^3/cm^3 and 1·06 cm^3/cm^3 respectively. The method used for determining this quantity was similar to the method used for determining the effective surface moisture content by Becker[2] and will be briefly outlined in the next section.

The estimated sphericities were approximately 0·91 for Ponca wheat and 0·84 for Seneca wheat. The value of the sphericity

of Ponca wheat, 0·91 was identical with that estimated by Becker[1] for Thatcher wheat.

The values of the volume to surface-area ratio, V/S, for Ponca and Seneca wheats were 0·0547 cm and 0·0504 cm respectively. In correlating the experimental data, the value of V/S was treated as a constant.

Steeping water was taken from the city water system. The analysis of water was reported as follows.

Total hardness (parts of calcium carbonate per million)	76
Non-carbonate hardness (parts of calcium carbonate per million)	45
Total dissolved solids (parts per million)	218
pH	7·5–8·0

Twenty-gram samples of wheat were placed in wire gauze baskets and immersed in a stirred water bath controlled to within 0·5°C of the set temperature. At the end of each immersion period, each sample was quickly removed from the water bath and superficially dried on large filter papers, as suggested by Becker.[2] After the surface water on the wheat kernels was removed, the weight of the sample was determined, and the weight gain was calculated.

For the measurement of the volume of the wheat kernels, the liquid displacement method was used, with toluene as the liquid. After the weight of the sample was measured at the end of each immersion time, the sample was charged into a burette which was filled with a known amount of toluene. The displaced volume of toluene was taken as the volume of the sample.

The initial volume content was also measured by the liquid displacement method after the initial moisture content had been determined by a two-stage air-oven method.[12] Initial volume content was expressed, by analogy with the initial moisture content, as the volume of moisture (as liquid water) originally contained in the wheat per unit volume of dry wheat.

In order to evaluate the effective surface volume content, samples of Ponca wheat were prepared with initial volume contents ranging from 22·42 to 73·8 per cent (dry basis), and samples of Seneca wheat were prepared with initial volume contents from 18·42 to 51·8 per cent (dry basis). The samples were immersed in water at 30°C, and their final volumes were measured after 15 min of immersion.

For designing steeping vessels, a knowledge of the changes in bulk volume or porosity of the beds of cereal grains during the steeping is as important as, or even more important than, one of the change in true volume of the grain kernels. For the measurement of bulk volume and bed porosity, a large sample of 100 or 200 g was charged into a glass column through which water was circulated from the tank as shown in Fig. 1. The bed height and bulk volume of this large sample in the column were continuously recorded throughout each series of experiments. This made possible a comparison of bulk volume with true volume, which was obtained from small samples, and eventually made possible the evaluation of the porosity of the bed of wheat kernels.

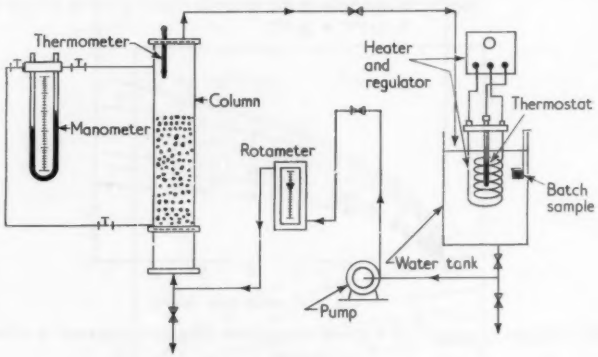

Fig. 1. Schematic diagram of equipment

Experiments were carried out over a range of temperature from 26·7°C to 60°C, and immersion periods ranged from several minutes up to eight hours.

Results and Discussion

The weight increase by surface-capillary action measured at various temperature levels shows that this weight increase is practically independent of temperature variation. The average initial moisture gain by surface-capillary action was 3 per cent (wet basis).

The results of this investigation on the rapid moisture pick-up at the onset of steeping are in agreement with those reported by previous investigators.[8, 10] This rapid moisture pick-up had been attributed to a capillary action by shallow pores in the pericarp, the outermost layer of the wheat kernel. Since this action is very rapid and ceases when the pores in the pericarp are filled with water, it is to be expected that volume change of the wheat kernels could not be detected at this stage of the water penetration.

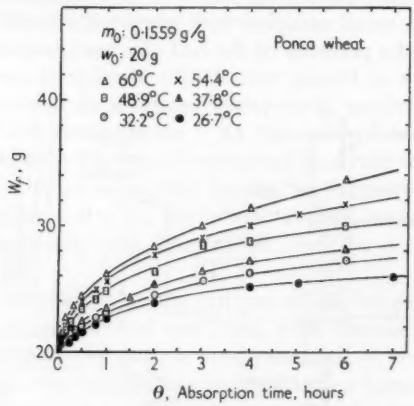

Fig. 2. Weight of sample at a given absorption time as a function of absorption time

The experimental data on weight and volume increases for Ponca wheat are graphically presented in Figs. 2 and 3 respectively. It appears that qualitatively the curves for weight increase in Fig. 2 and for volume increase in Fig. 3 are almost identical. This indicates that there may be a linear relationship between weight increase and volume increase.

If it is assumed that the volume increase of the wheat kernels is due to the formation of the empty spaces of the cracks inside the kernels, and that the penetration of water into the kernels is due mainly to the flow of water into the cracks formed, as observed by Grosh and Milner,[6] the following relationship can be written from the simple material balance:

$$\rho_f \sum \Delta V = \sum \Delta W \tag{7}$$

where ρ_f is the density of water, $\sum \Delta V$ the total volume increase, and $\sum \Delta W$ the total weight increase.

Close examination of the data showed that the values of $\sum \Delta W$ were consistently greater than those of $\rho_f \sum \Delta V$. This may be explained by the fact that the initial weight increase by the rapid capillary action did not cause the volume increase, as mentioned previously. Therefore, in Fig. 4 the relationship represented by equation (7) is plotted using the experimentally obtained data after the average initial weight increase due to the capillary action has been subtracted from the total weight increase. Fig. 4 positively indicates that the relationship given by equation (7) holds up to a very high degree of water absorption.

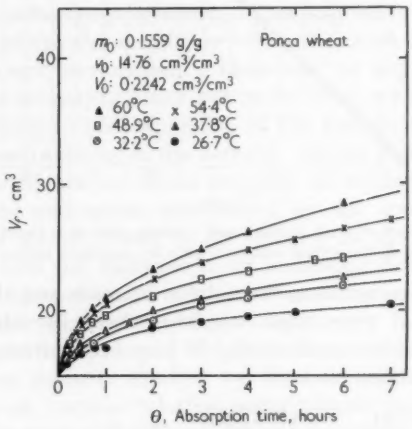

Fig. 3. Volume of sample at a given absorption time as a function of absorption time

The data given in Fig. 4 were analyzed statistically. First, the sample correlation coefficient of Fig. 4 was found to be 0·9994. This value signifies a good linear correlation between the product of water density and the total volume change, and the total weight change. Next, the hypothesis that $\beta = 1$, where β is the slope of the regression line, was tested against b, which is the sample regression coefficient. The sample regression coefficient

for Fig. 4 was 0·998. The hypothesis, $\beta = 1$, was accepted at the 0·01 level and also at the 0·05 level.[11] The test of the hypothesis definitely indicated that the product of water density and the total volume change is identical with the total weight change.

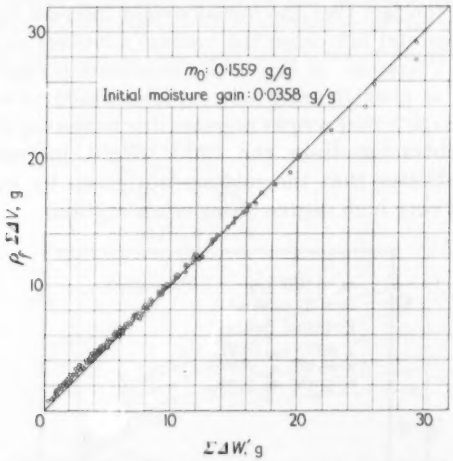

Fig. 4. The product of fluid density and volume gain as a function of the moisture gain from which the initial moisture gain by capillary action was subtracted

The difference between the volume increase and the volume of water that had penetrated into the wheat kernels during the immersion time was evaluated. It may be approximated by the following equations:

$$\delta = \frac{0{\cdot}7194}{\rho_f} - \frac{0{\cdot}04726 \sum \Delta W'}{\rho_f} \quad \text{for Ponca wheat} \tag{8}$$

$$\delta = \frac{1{\cdot}730}{\rho_f} - \frac{0{\cdot}192 \sum \Delta W'}{\rho_f} \quad \text{for Seneca wheat} \tag{9}$$

where δ is the difference between the overall volume increase and the volume of water absorbed by the wheat kernels after the initial capillary action, and $\sum \Delta W'$ is the overall weight increase minus the average moisture increase by capillary action. The equations indicate that these differences were indeed small within the experi-

mental range. The close linear relationship between the volume increase and the volume of water absorbed by the wheat kernels may well be explained by the mechanism of water penetration proposed by Grosh and Milner.[6] They reported that the formation of both transverse and radial cracks is initiated inside the wheat kernels immediately after the capillary absorption of water by the pericarp. The penetration of water into the kernels may be mainly due to the flow of water into the cracks formed.

In spite of the close agreement between the volume increase and the volume of water absorbed, equations (8) and (9) definitely indicate that the volume increases were greater than the volumes of water which penetrated at the earlier stage of water diffusion. The values of the volume increase diminished continuously, and eventually became smaller than the volume of penetrated water as the water diffusion progressed. This may be explained by the observation of Grosh and Milner[6] that the crack formation preceded the water penetration. Therefore, at any instant there could still be an empty crack yet to be filled with water (which only contributes to the expansion of the kernels but not to the weight increase) existing in the kernels. As the water penetration and formation of internal cracks progress, the contact area between water and the endosperm surrounded by the cracks should increase. This should result in an increase in the rate of water penetration into the endosperm by molecular diffusion. Grosh and Milner[6] reported that crack formation becomes less significant when the moisture content of wheat is high.

The changes in volume and weight of wheat with gain or loss of moisture were recently studied by Bushuk and Hlynka[3] after equilibration at various relative water-vapour pressures. Contrary to our investigation, they reported that initially the weight change is faster than the corresponding volume change, and at high moisture levels the volume change is faster than the weight change. Perhaps, this contradiction of results may arise from the fact that the experiments were conducted under completely different conditions. In one the changes in weight and volume of the wheat kernels were measured at the instantaneous conditions and the other was conducted at equilibrium. In spite of the contradiction of the results in the above respect, the differences between the weight and volume changes are so small that it is quite

reasonable to assume that the volume change of the wheat kernels is directly proportional to the weight change during the steeping operation. In view of this, the equation derived by Becker[1] can be applied to the volume increase.

The bulk volume and porosity of the bed of wheat kernels are graphically presented in Figs. 5 and 6. While the bulk volume increased continually as the steeping progressed, the porosity remained constant and independent of temperature variation and steeping time as shown in Fig. 6. The average value of porosity was 0.45. It can be seen from the method of steeping that the actual sizing of the steeping vessels must not be based on the true volume of the wheat kernels but rather on the bulk volume of the beds of wheat kernels. Fig. 5 indicates that the increase of bulk volume was quite appreciable. Since the increase in bulk volume can be related to the increase in true volume of wheat kernels for the same amount of sample by $\sum \Delta V/(1-\epsilon) = \sum \Delta V_b$, where ϵ is the porosity, and $\sum \Delta V_b$ the total bulk volume change, the increase in bulk volume should be approximately $1/(1-\epsilon) = 1/(1-0{\cdot}45) = 1{\cdot}8$ times greater than the increase in true volume. This is borne out by the data obtained.

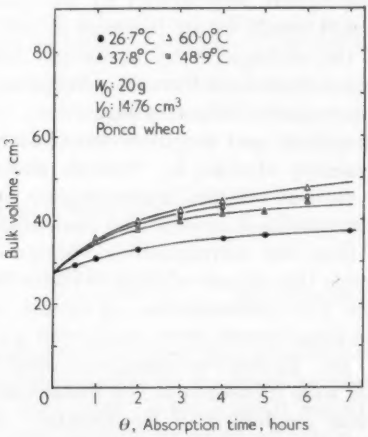

Fig. 5. Bulk volume as a function of absorption time

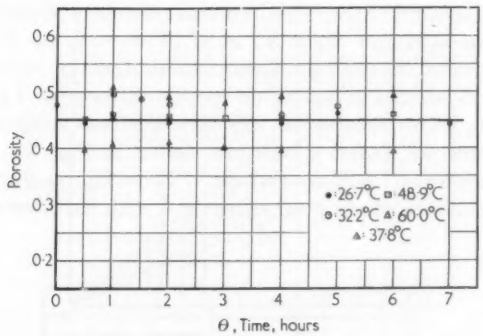

Fig. 6. Porosity of the bed of wheat kernels as a function of time

In addition to the true volume of the wheat kernels, the bulk volume, or more strictly speaking the height and the porosity of the beds of wheat kernels, is also a determining factor for the pressure drop in the water circulated through the beds during the steeping operation when the water temperature and flow rate are kept constant. As indicated previously, the porosity of the beds was found to remain practically constant. The bulk volume (or bed height) and the true volume of the wheat kernels, however, have opposite effects on the pressure drop of fluid flow through the beds. Therefore, the pressure drop will not change appreciably during the steeping operation. Several data, summarized in Table I, were obtained to illustrate this.

Table I. Pressure drop of fluid flow through the beds

Temp., °C	Flow rate, lb/h ft²	Time, h	P (Pressure drop), lb/ft²
47	12,520	1	4·29
		2	4·05
		3	4·05
		4	4·05
		6	4·07

In evaluating the effective surface volume content, equations (5) and (6) show that if the coefficient of volume increase is independent of volume content in the range studied, $K_v(V/S)$ should

be a linear function of the initial volume content and should extrapolate to an intercept $V_0 = V_s$ at $K_r(V/S) = 0$, or $\bar{V} - V_0$, the volume increase, should be a linear function of the initial volume content and should extrapolate to an intercept $V_0 = V_s$ at $\bar{V} - V_0 = 0$. The results of effective surface volume content measurements for Ponca wheat are shown in Fig. 7. A similar figure was obtained for Seneca wheat. These figures show that the experimental data are in good agreement with the above relationship.

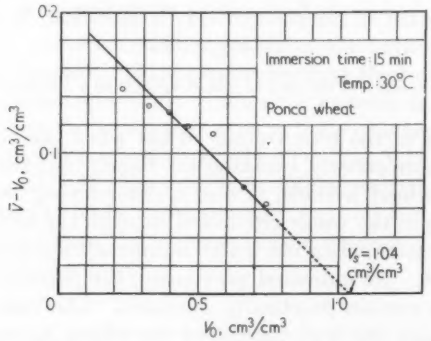

Fig. 7. The volume increase as a function of the initial volume content

The experimental data for Ponca wheat are plotted in Fig. 8 using the relationship represented by equation (6). A similar figure was obtained for Seneca wheat. Both figures confirmed that such a relationship exists. The ranges of validity, however, were different for Ponca wheat and Seneca wheat. It was smaller for the latter, especially at higher temperatures. The slopes of the curves in both figures were obtained by the method of least squares. The results are summarized in Table II. The curves in Fig. 8 for Ponca wheat and a similar figure for Seneca wheat extrapolate to zero volume increase, while similar curves for the weight increases extrapolate to an intercept, $\bar{m}_i$, corresponding to the moisture pick-up by capillary action.[2] This may, however, indicate that the rapid moisture pick-up by capillary action by pores of bran does not, as would be expected, cause the volume of the wheat kernels to increase.

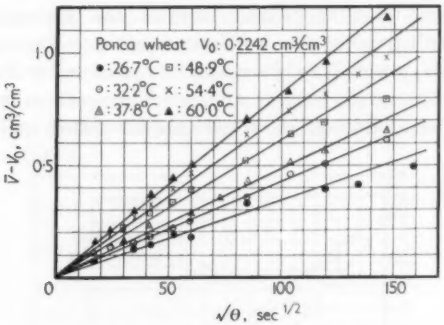

Fig. 8. The linear relation between the volume increase and the square root of time

Table II. Slopes of linear regression lines for $(\bar{V}-V_0)\,vs.\,\sqrt{\theta}$ and $(\bar{m}-m_0)\,vs.\,\sqrt{\theta}$

Temp., °C	$K_v \times 10^3$,[a] cm³/cm³ sec$^{\frac{1}{2}}$	$K_m \times 10^3$,[a] g/g sec$^{\frac{1}{2}}$	$K_v \times 10^3$,[b] cm³/cm³ sec$^{\frac{1}{2}}$	$K_m \times 10^3$,[b] g/g sec$^{\frac{1}{2}}$
26·7	3·457	2·050	3·9	2·7
32·2	4·359	2·626		
37·8	4·826	3·086	5·0	3·6
48·9	6·077	4·027	7·0	5·3
54·4	7·113	4·664		
60·0	8·236	5·367	9·1	6·9

[a] Ponca Wheat.
[b] Seneca Wheat.

The coefficients of volume change, D_v, can be evaluated from equations (5) and (6) if the effective surface volume content is known:

$$D_v = \left[\frac{\sqrt{\pi}}{2}\frac{V}{S}\frac{K_v}{(V_s - V)}\right]^2 \tag{10}$$

Fig. 9 shows coefficients of volume change calculated from the experimental data as a function of the reciprocal of the absolute temperature. Fig. 9 indicates that the coefficients of volume change obey the Arrhenius relation, $D_v = D_0 \exp(-E/RT)$, in which E is the activation energy, R the universal gas constant,

and T the absolute temperature. The constant D_0 and the slope, $-E/RT$, of the linear regression line of the Arrhenius relation were estimated by the method of least squares. The values of the activation energy obtained were 10,129 cal/mole for Ponca wheat and 11,030 cal/mole for Seneca wheat. The coefficients of volume change for Ponca and Seneca wheats are tabulated in Table III.

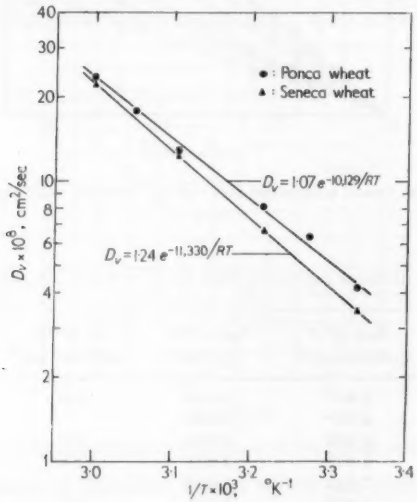

Fig. 9. The coefficient of volume increase as a function of the reciprocal of absolute temperature

Table III. Coefficients of volume change

Temp., °C	Ponca wheat $D_v \times 10^8$, cm²/sec	Seneca wheat $D_v \times 10^8$, cm²/sec
26·7	4·12	3·42
32·2	6·54	
37·8	8·02	6·66
48·9	12·72	12·70
54·4	17·42	
60·0	23·36	22·20

The constants, D_0, for Ponca wheat and Seneca wheat were 1·068 cm²/sec and 1·24 cm²/sec respectively. The coefficients of volume change and the activation energies evaluated from the present data fall within the range of the diffusion coefficients and the activation energies found by Becker[2] from diffusion studies. The linear regression lines in Fig. 9 are in close agreement with the Arrhenius relation.

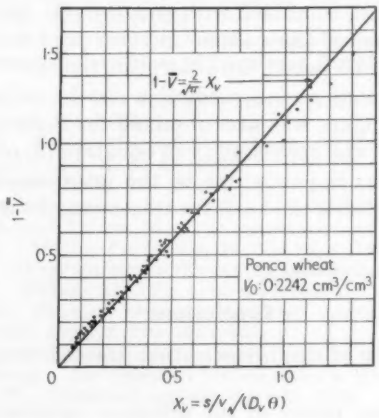

Fig. 10. Plot of all the data of Ponca wheat on volume increase according to equation (5)

The weight-gain data obtained in this study were analyzed and correlated according to Becker's method.[2] The Arrhenius relationships between the diffusivity, D_m, and temperature for Ponca and Seneca wheats are given as follows:

$$D_m = 4{\cdot}65\, e^{-11{,}231/RT} \quad \text{for Ponca} \tag{11}$$

$$D_m = 2{\cdot}618 e^{-11{,}930/RT} \quad \text{for Seneca} \tag{12}$$

These results are comparable to that obtained by Becker.[2]

Fig. 10 shows the general relation, equation (5), of all the data of Ponca wheat on the volume increase for temperature in range of 26·7°C to 60°C. The parameters were calculated from the experimental relations

$$1 - \bar{\bar{V}} = (\bar{V} - V_0)/(V_s - V_0)$$

$$V_s = 1{\cdot}04 \text{ cm}^3/\text{cm}^3 \quad \text{for Ponca}$$

$$V_s = 1{\cdot}06 \text{ cm}^3/\text{cm}^3 \quad \text{for Seneca}$$

$$D_v = 1{\cdot}068 \exp(-10{,}129/RT) \quad \text{for Ponca}$$

$$D_v = 1{\cdot}24 \exp(-11{,}030/RT) \quad \text{for Seneca}$$

The above relations, combined with equation (5), can be used for practical prediction of the volume increase as a function of the independent variables, which are absorption time and temperature. Fig. 10 indicates that such a prediction can be made with accuracy. A similar figure was also obtained for Seneca wheat. As noted by Becker [2], the applicability of equation (5) over the entire experimental range is partly due to the prior assumption of its validity made to define the basis for treatment of the data.

Conclusions

From the results of this investigation, the following conclusions may be drawn:

(1) A Fick's law model of the diffusion equation in the form obtained by Becker may be used to represent the volume increase of the wheat kernel at the initial period when it is immersed in water.

(2) The range of applicability of this model may depend on the variety of wheat.

(3) The moisture increase due to capillary action does not cause the volume increase of the wheat kernels.

(4) The volume increase and weight increase, when wheat kernels are immersed in water, are closely related to each other. The relationship is almost linear.

(5) This fact may indicate that the mechanism of water penetration proposed by Grosh and Milner [6] is highly probable for both hard and soft wheat at the initial period of water penetration.

(6) Comparisons between the volume and weight increases of wheat kernels may be used to characterize the types of wheat.

(7) The Becker equation for the weight increase is valid.

Acknowledgement. The helpful suggestions received from Dr. Max Milner and Mr. Gorden M. Grosh at the early stage of this study are deeply appreciated.

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Chemical Separation Methods for Common Microbes

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Summary. To collect microbes from suspensions by a process other than centrifugation, it has been found effective to add inorganic and organic electrolytes in the colloidal state. The addition of electrolytes causes a neutralization of charges on the particles in suspension resulting in their coagulation and precipitation. High-molecular-weight compounds which form micelles also promote coagulation and precipitation. It is believed that chemical collection is an extremely efficient method of treatment for the mass production of microbial suspensions.

Introduction

Industrial practices up to the present time normally include centrifugation to separate microbes from their culture media. Centrifuges such as the 'Sharples' and 'De Laval' are commonly used. However, such physical separation methods do not seem very effective when dealing with very large quantities of microbial suspensions.

The author has done research for the past few years, in contrast to physical separation methods, on chemical separation methods.[8] Methods of collecting microbes by chemical separation were almost unknown up to the present time. In short, the process utilizes chemical agents to separate suspensions of microbes by means of coagulation and precipitation.

A considerable amount of research has been done to date on the chemical treatment of contaminated water such as sewage and industrial waste. For example, Reynolds,[9] Schroeffer,[12] Agar,[1] Rudolfs,[10] Lloyd,[5] Evans and Shrey,[2] Hirose,[3] etc. reported on the chemical purification of sewage. It has been reported by Ruewin and Ward,[11] Michael,[6, 7] etc. that recently developed high-polymer chemicals, including some special high-polymer compounds, contain electrolytes which cause coagulation and precipitation of particles of soil dispersed in contaminated water. Johnson[4] recently reported the effect of a polycationic high polymer as a coagulation agent for water purification.

It is understood that such inorganic and organic electrolytes effect coagulation and precipitation of fine soil particles in water that is commonly contaminated with sewage and industrial waste. However, it still seems unestablished whether or not these electrolytes effect the coagulation and precipitation of common microbes.

The author has studied the reaction on coagulation and precipitation of common microbial suspensions, using various inorganic and organic electrolytes.

Unlike agents used for coagulation and precipitation with ordinary contaminated water, chemicals to be used for separating microbes must possess the following requirements:

(1) They must react rapidly.

(2) They must be non-toxic.

(3) They should not alter the chemical constituents of the cells.

(4) No coloration should be developed due to the addition of the chemicals.

(5) Chemicals should have the minimum cohesive power. (Water cannot be extracted effectively with a filter-press if the chemical has strong cohesive powers.)

(6) Neither high acidity nor high alkalinity should result upon the addition of chemicals.

(7) The quantities of chemicals must be as small as possible and highly effective.

(8) Prices of chemicals to be used should be reasonable for large-scale use.

(9) It is desirable that these chemicals be completely removed by washing after being used.

(10) It is desirable to recover the chemicals for re-use after washing.

In view of these requirements, most of the electrolytes currently used for the deposition of sewage, industrial waste, etc. do not seem satisfactory for use in separating microbes. For example, aluminium electrolytes used for the purification of service water and sewage do not seem favourable because of the comparatively large amounts needed, neither do iron electrolytes due to their colour revelation.

The author has experimented with chemicals which seemed suitable according to these requirements and has discovered two or three effective precipitation agents, as reported below.

Methods

A jar-test method was adopted for the experiments. Microbial suspensions were placed into beakers and small amounts of coagulation and precipitation agents were added. The purpose of this experiment was to see whether or not perfect separation of microbes and liquid could be observed.

One litre of suspension collected from the culture tank was measured into each beaker. The chemicals to be tested were then introduced into the suspensions in the beakers. After a minute of gentle mixing, the suspensions were allowed to stand and it was observed whether or not complete separation of microbes was effected.

Microbes which were industrially available in large amounts were selected, e.g. yeast (*Saccharomyces*, *Torulopsis*, etc.), bacteria (*Bacillus subtilis*, *Lactobacillus*, etc.), and microalgae (*Chlorella*, *Scenedesmus*, etc.).

Experimental Results

1. *Effect of common inorganic electrolytes.* The following aluminium compound electrolytes were observed for their effectiveness in coagulating and precipitating microbes from aqueous suspensions:

Aluminium sulphate, $Al_2(SO_4)_3$; aluminium oxide, Al_2O_3; aluminium hydroxide, $Al(OH)_3$; alum, $KAl(SO_4)_2 . 12H_2O$; $Al_2Cl_2SO_4(OH)_2$; $Na_2Al_2O_4$; etc.

Two of the above compounds showed notable reactions. They were aluminium sulphate and alum. However, quantities used for these experiments were comparatively large, 1–2 per cent, and foam was observed in some cases when the chemical was added. Considering these results, the above compounds do not seem suitable as separating agents for microbes.

Observations were also made using the following iron-compound electrolytes:

Ferrous chloride, $FeCl_2$; ferric chloride, $FeCl_3$; ferrous sulphate, $FeSO_4$; ferric sulphate, $Fe_2(SO_4)_3$; etc.

Ferric salts were found to be very effective. When these iron-compound electrolytes were used in combination with calcium hydroxide, their reactions were especially notable. However,

iron-compound electrolytes impart a light brownish colour to microbes and therefore do not seem favourable for use as separating agents.

Other inorganic electrolytes which showed some reaction were sodium silicate, Na_2SiO_3; cupric chloride, $CuCl_2$; magnesium chloride, $MgCl_2$; zinc chloride, $ZnCl_2$; sodium fluoride, NaF; etc. When they were used in combination with 1–2 per cent of calcium hydroxide, notable reactions were observed. However, when these chemicals were added, the volume of precipitate increased excessively.

It is rather interesting to note that calcium-compound electrolytes used in large quantities are moderate in price and completely harmless. Chemicals such as calcium oxide, CaO; calcium hydroxide, $Ca(OH)_2$; and calcium chloride, $CaCl_2$, were tested. Calcium hydroxide and calcium chloride were found to be more effective than the other compounds. It is considered that the effectiveness of the coagulation and precipitation reactions shown by calcium salts is due to calcium ion. However, the reactions also indicated that notable effects were due to the hydrogen ion concentration in the microbial suspension. Furthermore, reaction in the acid state was not as pronounced as it was in the normal alkaline state. The disadvantage of calcium electrolytes is the comparatively large quantity needed (1·5–2 per cent). The volumes of precipitates were increased when these chemicals were added.

2. *Effect of coagulation and precipitation by calcium salts.* Among the calcium salts, the compound which caused the most pronounced coagulation and precipitation in the microbial suspension was calcium chloride. However, the reaction took place in the alkaline state. For this reason, 0·2–0·8 per cent of sodium hydroxide or potassium hydroxide was added to the microbial suspension to keep the hydrogen ion concentration at pH 8·0–9·5. Calcium chloride (0·1–0·5 per cent) was added and the suspension was mixed slowly. In this process, microbes in suspension will rapidly gel and begin to coagulate and precipitate. At this stage of processing, the following chemical reaction occurs:

$$2NaOH + CaCl_2 = 2NaCl + Ca(OH)_2 \qquad (1)$$

It is believed that microbes are adsorbed by the calcium hydroxide, resulting in coagulation and precipitation. An initial addition of calcium hydroxide only will not result in any notable coagulation and precipitation in this case.

Since the sediment obtained by the above experiment contained calcium hydroxide, 30–60 minutes later the sediment coagulated into a pasty form. Filtration of such coagulated sediments can be readily performed.

If this solid sediment is washed with 0·05–0·5 per cent of dilute hydrochloric acid, the calcium hydroxide is dissolved, and the pH changes, leaving only the microbes.

$$Ca(OH)_2 + 2HCl = CaCl_2 + 2H_2O \qquad (2)$$

Calcium chloride formed at this stage may be collected for re-use. Furthermore, the salt formed by reaction (1) can be removed by a water-washing process.

By the method described above, the Japan Microalgae Research Institute, which produced 10 tons of microalgae suspension in a day's operation, found it possible to collect the microalgae (*Chlorella* and *Scenedesmus*) in a very efficient manner.

3. *Effect of coagulation and precipitation by titanium salts.* The author previously found other chemicals which reacted strongly in the development of coagulants for suspensions of common microbes. These chemicals were titanium tetrachloride, $TiCl_4$, and titanium trichloride, $TiCl_3$.

Titanium tetrachloride in concentrated solution gives off fumes. It is light green in colour when diluted 50 per cent with water, and becomes easy to handle and is suitable as a stock solution. The concentration used should be approximately 0·01–0·02 per cent. The 50 per cent solution should be diluted to this concentration just prior to its application. The dilute solution is colourless.

Significant coagulation and precipitation can be observed with titanium tetrachloride and titanium trichloride even in very small quantities (approximately 0·01 per cent). The addition of 0·01 per cent titanium tetrachloride to suspensions of yeast, bacteria, microalgae, etc., while stirring slowly, results in immediate, marked coagulation of microbe-containing flock, which

precipitates rapidly. Extraction of water in the precipitated flock can be easily accomplished with a filter-press.

There is another interesting point with regard to titanium tetrachloride. This chemical, when heated to more than 136°C, vaporizes and forms titanium tetrachloride gas. When titanium tetrachloride gas is jetted into a microbial suspension in the separating tank, coagulation and precipitation ensue. After titanium tetrachloride gas (0·01–0·02 per cent as a water solution) was jetted into 100 l. of microalgae (*Chlorella ellipsoidea*), approximately 15–20 min was required to observe complete separation of algal cells.

When titanium tetrachloride gas is dissolved in water, dissociation occurs as follows:

$$TiCl_4 \text{ (in the form of gas)} \rightarrow TiCl_4 \text{ (liquid)} \rightarrow Ti^{4+} + Cl^-$$

Titanium ion, as a polyvalent ion, can neutralize the electric charge on micro-organisms in suspension, resulting in coagulation and precipitation of the cells.

4. *Effect of coagulation and precipitation by large-molecule electrolytes.* It is well known that glue, sodium alginate, polyacryl acid polymer, cationic surface-active agents, etc. are suitable large-molecule electrolytes. However, the adhesive power of glue or sodium alginate is so strong that it interferes with the water-extraction stage. Polyacryl acid polymer gave a reaction, but when used independently its reaction speed was slow due to its strong adhesive power.

The author has been able to promote the reaction by using a combination of polyacryl acid polymer and calcium chloride. Coagulation can be observed with calcium chloride alone when the suspension is alkaline. However, this reaction can be markedly promoted by the addition of polyacryl acid polymer, using 0·1–0·4 per cent calcium chloride and 0·1–0·2 per cent polyacryl acid polymer.

As another result of these experiments, the author has discovered that cationic surface-active agents such as alkyl pyridinium salts, quarternary ammonium salts, alkyl amines, etc. give effective reactions as chemical separating agents using dosages of 0·01–0·1 per cent.

Alkyl radicals with few carbon atoms show high solubility but low coagulation reactions, while lower solubility and higher coagulation reactions occur as the number of carbon atoms is increased. The author has experimentally demonstrated that alkyl radicals with up to 12–18 carbon atoms were effective and has also found the following to be effective:

Dodecyl ammonium chloride, dodecyl amine chloride, dodecyl trimethyl ammonium chloride, hexadecyl trimethyl ammonium chloride, cetyl pyridinium chloride, cetyl oxymethyl pyridinium chloride, stearamido methyl pyridinium chloride, octadecyl pyridinium chloride, dodecyl pyridinium chloride, cetyl oxymethyl pyridinium chloride, etc.

Bromides of high-molecular compounds also demonstrated some effectiveness.

These surface-active agents give slow reactions but they require very small dosages and yield lower volumes of sediment. Also, they do not possess the cohesive power of the polyacryl acid polymer and for this reason water extraction by the filter-press can be performed simply and profitably.

5. *Principle of coagulation and precipitation by chemicals.* Microbial suspensions contain large amounts of cells in colloidal form during cultivation. Each cell is dispersed during growth and is electrically negative in charge. For this reason, microbes mutually repel each other during their growth. Their colloidal form makes them rather difficult to observe during precipitation. It is also believed that dispersed microbes usually contain alkali, but they may also contain acid or salt, depending upon conditions.

The suspension of microbes is not in a simple colloidal form. Its formation is complicated owing to the products of metabolism and the emission of carbon dioxide, etc. Furthermore, in suspension, individual microbes acting as shelters for hydrophilic colloids make observation of any natural means of precipitation much more difficult.

In order to coagulate and precipitate microbes and separate them chemically, it becomes necessary to apply electrolytes to neutralize the negative charges on the cells.

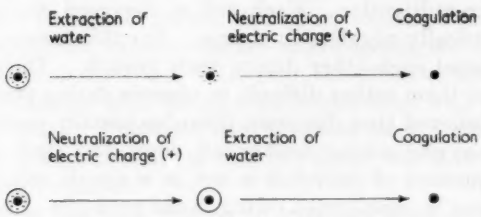
In a simple hydrophilic colloid, electric charges on the separate particles must be neutralized in order to observe precipitation. Therefore, it is usually only necessary to apply a small amount of electrolyte to see a precipitation reaction. However, in a

microbial suspension, a complicated hydrophilic colloid, neutralization of the electric charge alone will not show a marked precipitation reaction in many cases.

The affinity of microbes for water is very strong. Numerous water molecules are strongly attracted to microbes, forming a hydration layer. Furthermore, due to the electric charge upon the microbes, it is very difficult to effect coagulation without removing water molecules. Each microbe has been segregated by this layer of water, making it impossible to form secondary particles by direct contact.

For this reason, it becomes necessary to apply two-stage processing methods in order to observe coagulation and precipitation in a microbial suspension. The first stage is to remove the hydration layer and the second is to neutralize the electric charges. The process may be performed in the reverse manner, first by neutralizing the electric charges and secondly by removing the hydration layer.

The relations of the above reactions are shown in the following diagram:



There are some simple hydrophilic colloids that can be easily coagulated by the addition of a water-removal agent together with small amounts of electrolytes. In a microbial suspension, a complicated hydrophilic colloid, it is desirable to have certain kinds of electrolytes which serve to neutralize the charge and extract water, removing the hydration layer at the same time.

As for electrolytes which react effectively on the microbial suspension upon the addition of an already charged hydrosol, using polyvalent cations which contain strong electrical neutralizing

agents (for example, quadrivalent titanium ion, etc.), it is possible to convert the sol rapidly into a gel. Needless to say, there will be no effect on the anion in this case.

When strong cationic agents are added to microbes, they immediately lose their charge and coagulate forming a strong absorption film. This coagulation sometimes shows a peculiar section. Simultaneously, other sections form micelles and promote the coagulation reaction. Furthermore, among the high-molecular electrolytes, activated energy is in proportion to molecular polymerization. For this reason, in a dispersed system of polymolecules, such as high-molecular electrolytes, coagulation is stronger in a long molecule chain. In other words, the higher the degree of polymerization, the stronger is the coagulation effect.

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The Disintegration of Bacteria and Other Micro-organisms by the M.S.E.-Mullard Ultrasonic Disintegrator

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Summary. Two commercially available ultrasonic instruments are described, in which micro-organisms and other cells may be disintegrated.

Sound waves (20 kc/s) are generated in the cell suspension by means of titanium velocity transformers (probes) coupled to a magnetostriction transducer. One instrument operating at 500 W will disintegrate from 2 to 500 ml, the other operating at 50 W from 1 ml to 30 ml.

Yeast was used as a test organism but results on some other organisms are also given. Cell rupture is shown to be independent of sonically generated free radicals, but enzyme inactivation (alcohol dehydrogenase) is accelerated by free radicals. Increasing the viscosity, decreasing surface tension of the suspending medium, or the presence of CO_2 decreases disintegration. The addition of solid nuclei such as powdered glass, as well as small air bubbles, increases disintegration.

This is consistent with disintegration being due to cavitation, but does not indicate the precise mechanism of cell rupture. Some effects of sonic disruption are compared with disruption by other methods.

Introduction

Preliminary experiments on the disintegration of micro-organisms with the Mullard Ultrasonic Drill (Model E 7682) showed that it can be used for disintegrating small volumes of cell suspensions (2–30 ml). This paper is concerned with the effects of the M.S.E. Cell Disintegrator, which was developed from the Ultrasonic Drill. It differs from most other sonic disintegrators in that sonic oscillations of 19–20 kc/s frequency are generated by means of a probe partly immersed in the liquid being treated. The design of the probes ensures that the maximum sound energy is produced from the relatively low electrical energy (50–60 W). In addition, it was found that similar probe systems could be used at higher powers and experiments are reported in which volumes of 2 to 400 ml of cell suspensions have been disintegrated

with probes operated by a newly designed 500-W generator. This larger instrument has also been used in preliminary experiments on continuous flow treatment of cell suspensions.

Experimental

Description of the Apparatus

The 50-W disintegrator consists of a generator (Fig. 1b) and a magnetostriction transducer (Fig. 1a). Ultrasonic oscillations of 19–20 kc/s are generated electronically and these oscillations are transformed to mechanical oscillations in the transducer. This consists of a stack of nickel laminations bonded together and clamped at the half-wave nodal point between the sides of U-shaped coils in which a polarizing current is induced; a velocity transformer (probe) is screwed into the half-wave stack. Stainless-steel probes of various sections were originally employed, depending on the volume of liquid to be treated; later, titanium probes were used to increase efficiency and to avoid erosion of the probe ends. The unclamped end of the probe is immersed for about 0·5 mm in the cell suspension to be treated, which may be held in an open-ended thick-walled glass test tube or tubes of a variety of materials cooled in a stirred bath of ice and water (Fig. 1c). Solutions may be treated aseptically in vessels equipped with a silicone-rubber seal through which the probe passes (Fig. 1c). A window-type transducer (Neppiras, 1960) cooled with running water was used with the 500-W generator and in a few experiments with a 2-kW generator. Two titanium probes (6·3 cm top diam., 3·2 cm bottom diam.; 5·8 cm top diam., 2·0 cm bottom diam.) were attached by a centrally placed screw to the steel end of the transducer. Just above the junction of the transducer and probe a lead zirconate crystal (Bush Ltd.) was firmly attached to the transducer by a bolt. A lead from the crystal was taken to a valve voltmeter (Advance Ltd.) and to an oscilloscope (Mullard type). The zirconate crystal behaves as a strain gauge, not accelerometer (Neppiras, 1960), and the voltage output is directly proportional to the amplitude of vibrations of the probe tip. The oscilloscope indicates wave form at the probe tip and changes markedly when cavitation starts.

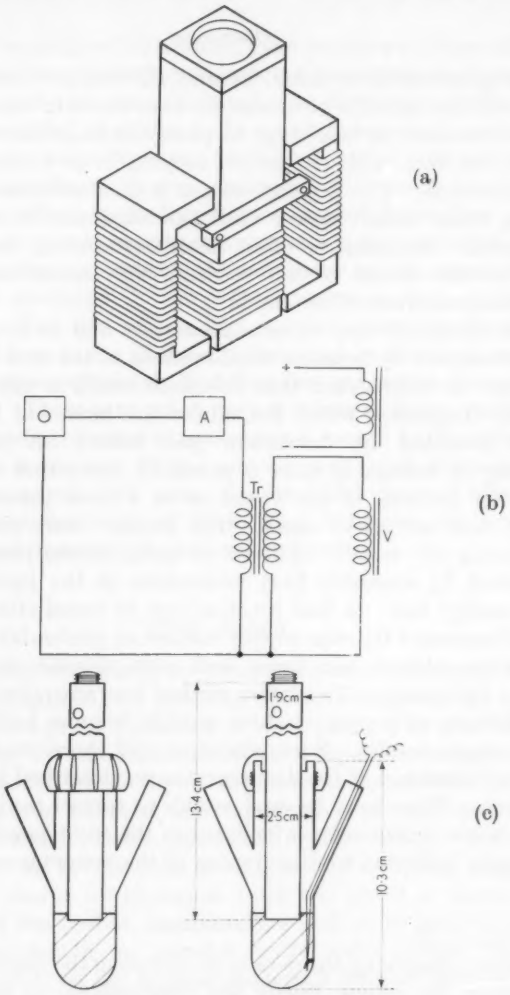

Fig. 1. Diagram of disintegrator

(a) Magnetostriction vibration transducer.
(b) Block schematic diagram of vibrator drive system. ± terminals for polarizing field; V, transducer; Tr, step-down transformer; A, power amplifier; O, variable frequency oscillator.
(c) Probe and vessel assembly for treatment of cell suspensions.

Operation

The generator is switched on and allowed to warm up for 10 min with the isolation switch on the transducer in the off position. It is convenient at this stage to place the suspension in position in the ice bath with the probe dipping *ca.* 0·5 mm below the liquid surface. The isolation switch is then switched on and the tuning knob turned slowly until the maximum hissing noise is produced. Retuning is often necessary during the treatment time as the release of cell contents may sometimes alter the acoustic properties of the liquid.

It is relatively easy to tune accurately and to detect faults in the transducer by listening to alterations in the note of the noise. It must be emphasized that the noise heard is not the note of 19 kc/s frequency, which few adults can hear, but is the mixed noise produced by cavitation (see below). A high pitched whining or screaming noise is generally due either to the probe touching the side of the vessel or to a loose junction between probe and half-wave stack. The former fault may result in shattering the vessel. A loose or badly fitting junction is also indicated by excessive heat production at the junction due to high energy loss. A bad junction can be cured either by fitting a well-annealed 0·2-mm copper washer or preferably by greasing the screw thread and faces well with silicone stoptap grease before tightening. This latter method has generally proved more satisfactory as copper washers quickly become brittle and then give erratic results. Some chemical and thermometric tests for over-all efficiency of the disintegrator are described in subsequent sections. These may be used as aids to correct tuning. In later models a microammeter is included in the front panel and correct tuning is indicated by the dipping of the ammeter needle.

Cavitation

Before describing the details of operating the disintegrator, it is necessary to outline briefly the phenomenon of cavitation, on which cell disintegration is known to depend (Noltingk and Terry, 1957). The passage of sound through liquid produces alternating pressure changes which, if the sound intensity is sufficiently great, cause cavities to form in the liquid. The

cavities, of the order of 10 μ diam., grow in size until they collapse violently with the production of high local velocities and pressures of the order of 1000 atm (Noltingk and Neppiras, 1950). It is generally assumed that the cells are disintegrated during the violent collapse stage (Nyborg and Hughes, 1961). In addition to disintegrating cells, cavitation produces a wide range of chemical and electrical effects in the liquid being treated (Lindström, 1956). In the case of water, sound pressures of 1–8 atm are needed (the so-called 'cavitation threshold') and these sound pressures are more easily produced in the 8–20 kc/s range than at higher frequencies. For this reason, the magnetostrictor system such as described here is one of the simplest ways in which to produce sound intensities sufficiently high to cause cavitation in bacterial suspensions. There is as yet no way in which to define accurately the intensity of cavitation or to measure it except in the attendant phenomenon it causes. In the present paper, cell disintegration has been compared with some chemical and thermal effects of cavitation, because both of these may influence the material obtained upon disintegrating micro-organisms.

Probe Design

In the present apparatus the production of sound energy in the liquid is accomplished by coupling the movement of the transducer stack to a probe immersed in the liquid. The efficiency of such coupling, i.e. the amount of the output of energy transformed into sound in the liquid, depends on the acoustic properties of the probe material and liquid; in fact, mainly on the impedance of sound in each material (Neppiras, 1960). The more closely these can be matched the higher the coupling efficiency will be. In almost all biological work the liquid is water; the dimensions of the probes, therefore, are such as to make as good an acoustical match as possible with water loads. Nickel, Monel-metal or brass are suitable materials for this purpose, but it was found that probes made of these materials eroded extremely rapidly at the ends and that they could not be protected by coating. Traces of Zn, Cu and Ni from such erosion are extremely undesirable in biological work and it was therefore decided to use stainless steel because, although the yield of acoustical energy

was lower, toxic effects due to erosion might be less. Two stainless-steel probes have been used for a great deal of this work, a 19-mm diam. parallel probe and a 'step down' probe with a top of 19-mm diam. and bottom of 6·3-mm diam. This latter probe increases the tip amplitude nine-fold (Table I). Such probes must be machined extremely carefully where the diameter changes, otherwise large losses of energy occur.

The efficiency of coupling has one other important aspect. Where loss of efficiency is due to mismatch in acoustic properties, the energy lost is generated as heat in the probe and transducer and is transferred from the probe to the liquid under treatment.

Table I. Dimensions of probes used as transducer–liquid couplers. In each case the amplitude given is the maximum expected. Under working conditions, this may alter depending on the liquid load and geometry

Material of probe	End diameters, mm	End ratios	Length, cm	Amplitude, μ
Brass	28–19	2 : 1	18·2	20
Brass	20–10	2 : 1	18·2	20
Brass[a]	17–2	—	19·5	*ca.* 60
Stainless steel	19–19	1 : 1	13·3	10
Stainless steel	19–9·5	4 : 1	13·3	40
Stainless steel	19–6·3	9 : 1	13·3	90
Titanium	30–20	1·5 : 1	12	15
Titanium	30–10	3 : 1	12	30
Titanium[b]	63–32	2 : 1	12	20
Titanium[b]	58–20	2·4 : 1	12	24

[a] Probe with exponential profile.
[b] Probes used in the 2-kW and 500-W instrument.

In order to reduce such heating effects and to avoid toxic effects due to erosion, probes of titanium (I.C.I. 318A) were tested (Table I). Very little erosion has been found with such probes and their disintegrative effects are better than those of stainless steel (cf. Tables).

Estimation of Cell Disintegration

Cell disintegration was usually measured by estimating the release of nitrogenous material from the cells under the following conditions: the suspension containing from 10 per cent to 1 per cent dry wt. cells was diluted five-fold and a sample taken for

estimation of total N by a micro Kjeldhal method. The rest of the sample was centrifuged for 10 min at $6000 \times g$ and a sample of the supernatant taken for total N determinations. These determinations gave the total N in the cell suspension and the total N in solution before treatment. Samples after treatment were diluted, centrifuged and the total estimation of N in the supernatant gave the amount of release of cell nitrogen by the treatment. In experiments with yeast in which total and broken cells were counted (Rodgers and Hughes, 1960), it was found that *ca.* 80 per cent of the total N of cells was released into solution when all the cells had been broken open. This was confirmed in experiments in ultrasonic disintegration where cell counts were also done (Table II). The release of 260 mμ absorbing

Table II. Release of soluble nitrogen and change in total cell count in yeast suspensions

Time, min	N in solution, % of total	Total cell count, $\times 10^5$/ml	Cells destroyed, % of total
0	5	12·8	0
5	26	10·4	18·8
15	55	4·0	69
28	65	1·8	86
35	80	0·5	96
50	84	1·6	87

20 ml of a 1 : 6 w/v suspension of yeast was treated for the times indicated in the 50-W instrument with the 19-mm parallel probe.

material or of protein by the trichloracetic acid method (Layne, 1959) was also tested but found not to be so satisfactory as the estimation of total N. Where it was inconvenient to measure total N, cell disintegration was measured by estimating the release of protein by the Folin (Layne, 1959) method or by the total dry weight of the $6000 \times g$ supernatants.

Iodine Formation from Potassium Iodide

The formation of free radicals during the passage of ultrasound through liquids has been shown to be due to cavitation. In quantitive studies of this phenomenon, Lindström (1956) has shown that the amount of free radicals, as indicated by the

formation of hydrogen peroxide or of iodine formation from potassium iodide, is dependent on cavitation intensity. As cell disintegration depends on cavitation, it seemed likely that some such estimation as suggested by Lindström might be useful in calibrating the ultrasonic disintegrators. The test finally used was a modification of that described by Weissler, Cooper and Snyder (1950). To 10 ml of 0·05 M KI was added 5 drops of 1 per cent soluble starch and 10 drops of carbon tetrachloride. The mixture was shaken and then treated for 5 min in the ultrasonic generator and the iodine formed was titrated with 0·01 N sodium thiosulphate. This test was found to be very reproducible and with the $\frac{3}{4}$-in. stainless-steel probes the titration ranged from 1·5 to 1·9 ml over a period of $2\frac{1}{2}$ years. With five different units tested at the M.S.E. factory, the averages of triplicates were 1·7, 1·8, 1·8, 1·9, 0·5 respectively. The low result was subsequently found to be due to a fault in the generator unit and when repaired it gave the same yield as the other instruments. Apart from using this test to study the relation of free radicals to cell disintegration, it was used routinely to calibrate and check each of the different probe systems. Some comparisons were also made with an older type 500-W Mullard generator.

Results

The Effect of Different Probes on Disintegration by the 50-W Disintegrator

The acoustic conditions in the 50-W disintegrator are varied by changing the end ratios of the probes, and by altering the volume of suspension. The effect of these changes on the disintegration of yeast (1 part yeast cake with 5 parts water) is shown in Table III. The parallel stainless-steel probe, which has the lowest end velocity, disintegrated more effectively in the smaller volumes (2 ml) and its efficiency decreased in the larger volumes. The two probes whose end velocities are greater than the parallel probe were both less effective in the smaller volumes but were more effective between 25–30 ml (Table III). Similar results were found with brass probes.

It is likely that the low efficiency of probes with highest end velocity in small volumes is due to the greater turbulence they

cause. For instance, when operating into 2 ml, the turbulence is so great that the liquid is thrown from the end of the probe, which for a great deal of the time then radiates into air instead of liquid. This situation is similar to that produced when large bubbles form during cavitation and it is called 'cavitation unloading'. Such unloading leads to a reduction of cavitation and increased heat production. Turbulence decreased with increase of volume. With the probes having smallest end velocities, turbulence even in the smallest volumes tested was generally barely sufficient to ripple the surface of the suspension; these probes were thus working at peak efficiency over the whole range of volumes tested. The decrease in percentage disintegration per ml of suspension (Table III) is expected if it is assumed that the phenomenon is of a 'single hit' type, and that the dosage applied is constant (Davies, 1959).

Table III. The effect of different probes and volume of suspension on the ultrasonic disintegration of yeast. A 1:6 w/v yeast suspension was treated for 10 min with the probes indicated in the 50-W instrument. The maximum temperature reached was measured with a thermocouple and soluble nitrogen released as described in the text

Probe size and material	Volume of suspension, ml	Temperature, °C	Nitrogen release, % total
19/19 mm, stainless steel	2	16·5	60
	5	15	30
	10	21	12
	25	12	2
19/9·5 mm, stainless steel	2	16	4
	5	16	9
	10	22	23
	25	19	45
30/20 mm, titanium	2	19	80
	5	16	45
	10	20	15
	25	19	8
30/10 mm, titanium	2	21	59
	5	14	38
	10	19	21
	25	21	19

The Effect of Power Level, Probes and Volume of Suspension in the 500-W Disintegrator

Preliminary experiments with a 2-kW generator showed that disintegration by probe systems as used in the 50-W instrument could be used with power levels up to 2 kW. It was considered from the disintegrative effects that a 500-W generator would be suitable for laboratory use and such an instrument would have design and economic advantages which outweighed the advantage of higher powered, larger instruments. The present instrument is the prototype of the M.S.E. 500-W disintegrator. The power input may be varied as well as probe size and Table IV shows the effects of these variables on the disintegration of different volumes of cell suspensions. Effective disintegration of 30 ml of yeast by the small probe was found with a power setting of 5 and this increases up to power input 7, when cavitation unloading becomes too great for the probe to be effective and rapid heating occurs. In smaller volumes of suspension (2–5 ml), breakage occurred at input levels as low as 0·5–1·0. In volumes of above 30 ml, disintegration may be increased by stepping up the power level above 7, but the results become erratic and heating more marked so that the larger probe is to be preferred in bigger volumes of solution (Table IV). In volumes of 160–400 ml, full power output may be used with the large probe without undue cavitation unloading (Table IV) but unloading and rapid heating take place increasingly as the volume is reduced below 160 ml.

Heat Production during the Ultrasonic Treatment of Suspensions

High local temperatures (*ca.* 10,000°K) have been predicted during the collapse stage of cavitation (Noltingk and Neppiras, 1951). Apart from the heat produced in solutions by losses of energy from the probes or adsorption of sound energy, which may be high in viscous solutions, extra heat is produced in cavitating systems, such as the present ones, and this is unavoidable if maximum rate of breakage is required. In the 50-W disintegrator, the over-all heat production in aqueous or dilute salt suspensions of cells raised the temperature from 22–25° to 50–60° in 5–10 min. By immersing the glass treatment vessels in stirred

Table IV. Yeast disintegration by the 500-W instrument

A 1 : 6 suspension of yeast was treated at the power level stated for 5 to 20 min.

Experiment A—Large Probe

Volumes from 20 to 80 ml were treated in a 100-ml beaker, 160 ml in a 250-ml beaker, 300 to 400 ml in a 500-ml Kilner jar.

Experiment B—Small Probe

The solutions were treated in a thick-walled 50-ml beaker for 5 min.

Experiment C

The suspension was treated with the small probe for the times stated in 30-ml amounts in a stout-walled glass tube.

	Volume of suspension, ml	Time, min	Power input (arbitrary scale)	Temperature, °C	Dry wt. in suspension, %
A	20	5	2	5	3·1
	20	5	5	5	4·2
	40	5	5	5	3·6
	40	5	5	5	3·0
	40	5	7	23	46·1
	80	10	10	25	40·6
	160	10	10	23	35·2
	300	10	10	23	21·3
	400	10	10	20	19·8
	400	20	10	20	32·4
B	2	5	5	22·4	59·2
		5	6	31·8	66·8
	5	5	5	19·4	37·2
		5	6	32·0	56·4
	10	5	5	15·0	26·8
		5	6	22·0	41
	15	5	5	32·2	35·4
		5	6	25·2	44·8
	20	5	5	21·0	21·6
		5	6	22·0	25·0
	30	5	5	18·2	14·4
		5	6	32·0	30·8
C	30	5	2	3	2·5
	30	5	3	5	3·2
	30	5	4	5	4·3
	30	5	4	5	4·2
	30	5	5	10	8·0
	30	5	5	10·8	13·0
	30	5	6	18	19·7
	30	5	7	25	42·7
	30	2	5	10·2	3·5
	30	5	5	10·2	8·0
	30	10	5	10·2	14·5
	30	15	5	10·0	28·7

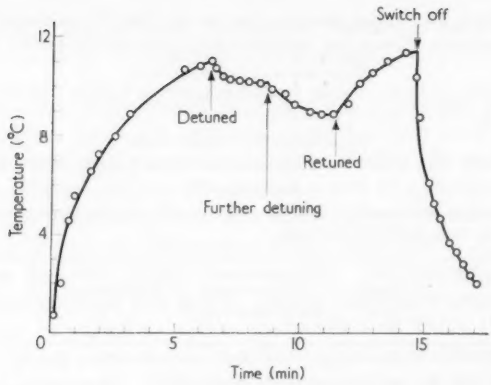

Fig. 2. The effect of tune on steady-state temperature. Twenty ml of yeast suspension (1 : 5 w/v) was treated with the 19 × 19 mm probe. The temperature was measured with the thermocouple, while the suspension was cooled in stirred ice-water

ice-water, temperatures were maintained between 5–18°. In stainless-steel vessels the temperature could be maintained at 5° but disintegration was markedly reduced (see page 420). More recently, lower temperatures have been maintained in titanium vessels without marked reduction of disintegration (Neppiras and Hughes, 1962).

With each particular probe, vessel and volume of suspension, the steady-state temperature, reached after about 2–3 min, was reproducible to within ±0·25° and was sensitive to alterations in the acoustic conditions, e.g. tuning. With the aid of an immersed thermocouple, the treatment could thus be followed by measuring heat production (Fig. 2). Thermometry has already been widely used as a means of ultrasonic dosimetry (Fry, 1958). Heat production by the 500-W instrument depends on the power input as well as the volume and nature of the liquid. The steady-state temperature permissible in any biological material must be established for each particular system. As a guide, some temperatures reached during the treatment of yeast suspension are given in Table IV.

In the experiments with viscous solutions (Table V), it was found that in the absence of stirring the temperature in the

region of the probe often reached 70–80°, whilst the bulk of the suspension remained at 5–6°. This must be especially guarded against when treating highly concentrated cell suspensions and bacterial suspensions rendered viscous by freezing and thawing. In this latter case, the high viscosity is probably due to highly polymerized DNA. By treating such material at low power levels while constantly gently stirring the liquid, the viscosity can be reduced in a few minutes without undue heating. The power level may then be increased to produce maximum disintegrative effects. Under such conditions the immersed thermocouple is extremely useful as a control of dosage.

Table V. The effect of sucrose and glycerol on yeast disintegration

Ten grams of yeast cake were suspended evenly in 50 ml of the solutions (1 : 6 suspension) and 30 ml treated for 5 min with the small titanium probe at power input 6. Protein was estimated in the supernatant after centrifuging for 20 min at 6,000 × *g*.

Sucrose conc., M	Temperature, °C	Protein in supernatant, mg/ml	
		Before	After
0·0	23	0·1	4·3
0·125	22	0·33	0·15
0·25	23	0·24	0·15
0·5	11	2·65	3·0
1·0	17[a]	3·0	2·5
2·0	12[a]	3·3	4·0
Glycerol conc., M	**Temperature, °C**	**Protein in supernatant, mg/ml**	
		Before	After
0·0	19	0·1	4·5
0·22	19	0·2	2·8
0·44	19	0·3	3·0
0·54	19·5	0·2	4·0
2·17	18	0·3	1·2
4·45	20[a]	0·3	0·5
10·1	[a]	3·0	3·0

[a] Intense local heating in region of probe, > 80°.

Table VI. The effect of ethanol and Tween 80 and NaCl on yeast disintegration

Ten grams of yeast cake were added to 50 ml of the solution (1 : 6 suspension) and 30 ml treated for 5 min with the small titanium probe at power input 6. Dry weight was estimated on the supernatants after centrifuging for 10 min at $6,000 \times g$.

Ethanol concentration, % w/v	Temperature, °C	Dry weight in supernatant, %
0	20	22·5
1·0	18	21·2
2·0	16	17·1
5·0	20	13·1
10·0	19	13·1
Tween 80 concentration, % w/v	**Temperature, °C**	**Dry weight in supernatant, %**
0·001	19	21·9
0·01	19·5	22·6
0·1	19·5	21·2
1·0	18·5	14·6
NaCl concentration, M	**Temperature, °C**	**Dry weight in supernatant, %**
0	19	29·0
0·125	19	16·9
0·25	19	21·2
0·5	20	10·2
1·0	21	9·4
2·0	21	14·4

The Effect of Suspending Fluid on Cell Disintegration

According to the equation of Noltingk and Neppiras (1950), either a rise in viscosity or a decrease in surface tension reduces cavitation intensity. The effect of viscosity on yeast disintegration was tested in solutions of sucrose and glycerol. Cell breakage was lowered as viscosity increased in the glycerol suspension; in the sucrose suspension cell disintegration was almost completely stopped in the lowest concentration, where the viscosity is not markedly different from that of water (Table V). It was noticed that all the sucrose suspensions evolved CO_2 and it is probable that the inhibition of disintegration is due to the cessation of cavitation caused by CO_2, as already reported by Prudhomme (1957). Passage of CO_2 through a yeast suspension during treatment also reduced disintegration.

The reduction of surface tension by the addition of Tween 80 (Atlas Powder Co.) or ethanol also decreased cell disintegration (Table VI). This effect is likely to be due to the decreased cavitation intensity as predicted by Noltingk and Neppiras (1950), but an additional effect of the surface-active agents on the outer cell layers cannot altogether be ruled out although, in general, surface-active agents tend to reduce the strength of cells (Putman, 1948) and would thus be expected to increase disintegration.

Increasing NaCl concentrations also decreased cell disintegration (Table VI). It is possible that this is due to increased cell rigidity due to the dehydration caused by the osmotic effect of the salt.

The Effect of Added Nuclei

In previous discussions on the effect of nuclei on cavitation, it was suggested that the addition of fine powders might increase cavitation (Bradfield, 1956). This was tried in narrow vessels where it has been shown that nucleation may be a limiting factor in cell disintegration (Table VII). Several powders, all of the order of 10–50 μ diam., considerably increased cell disintegration. The effect of embacel is noteworthy in that this material is a diatomaceous earth and each particle traps a considerable volume of air. The effect of making powdered glass non-wettable by treating it with silicone fluid slightly reduced its effectiveness.

Table VII. The effect of various powders on yeast disintegration

A 1 : 6 (w/v) yeast was treated with the 58–20 mm titanium probe and power input for 4·5 min. In Experiment A and in Experiment B, 10 ml of suspension was treated in a tube 3·5-cm wide and the liquid column was 1·5 cm deep; 0·1 g of powder was added in both cases.

Powder added	Temperature, °C	Dry wt. in super.	
		Expt. A	Expt. B
None	17	14·8	45
Norite	19	17·6	47·5
Hyflo supercel.	23	28·6	45
Powdered glass	23	28·3	51·5
Powdered glass[a]	23	26·1	—
Embacel	25	30·1	51

[a] Powdered glass had been treated with a carbon tetrachloride solution of silicone and then heated at 100° for 5 min to render it non-wettable.

Likewise Fluon (I.C.I. Ltd.), which is a non-wettable powder, was without effect. This does not agree with the prediction of Bradfield (1956) and although it is likely that the powders can by themselves act as cavitation nuclei, trapped air may also increase their effects.

The Effect of Shape of Vessel on Cell Disintegration

The shape of the vessel had no marked effect on the rate of disintegration with the 50-W disintegrator but affected markedly the rate of disintegration by the 500-W instrument. This was especially so when relatively small volumes were treated with high power, i.e. high electrical input and high amplitude. Disintegration of 30-ml yeast suspension treated in a glass vessel which had a wide air interface was higher than that treated in a vessel with no air interface. At the high power input used, it is likely that outgassing rapidly depleted the liquid of nuclei around which active cavities are thought to form and thus rapidly reduced cavitation intensity in the narrow vessel. In the wide vessel, air is rapidly brought in from the surface of the liquid; this moves across the face of the probe to the region of highest sound intensity where bubbles become active in forming intense cavitation streamers (Nyborg and Hughes, 1961).

In most experiments, glass vessels with stout walls (2–3 mm thick) and with rounded bottoms have been used. Thin-wall vessels (0·3–0·2 mm thick) often shatter, especially if the bottom is not rounded. Nylon or polythene centrifuge tubes are also satisfactory when cooled in ice water. Without surrounding water to act as a damping system, disintegration in these less rigid containers is less effective; likewise in stainless-steel tubes and beakers which were tested in order to improve cooling, disintegration was reduced by as much as 50 per cent.

Free-Radical Formation by Various Probes

With the 19×19 mm stainless-steel probe, free-radical formation as measured by the iodine formation from KI (see page 411) was independent of the concentration of KI in concentrations from 0·5 M to 0·02 M but decreased as the concentration was reduced to 0·01 M; it was still detectable in 0·001 M. The amount

of CCl_4 made no difference to iodine formation so long as some remained undissolved at the end of the reaction. The addition of starch to the reaction mixture rather than its addition after the treatment raised iodine yields by 10 to 15 per cent, probably because in its absence some iodine was lost by degassing. Under the standard conditions, iodine production was linear with time up to 20 min. In experiments run longer than this, the rate of iodine production decreased progressively and a red colour was produced, indicating a breakdown of the starch. Free-radical formation was highest with the probes having least amplitude and least with those having greatest amplitude (Table VIII).

Table VIII. Iodine formation by various probes

In each test the container held 10 ml of the standard reagents into which the probe dipped 0·5 to 1·0 mm below the surface. No external cooling was applied and each test was run for 10 min.

Probe	Iodine formation, ml 0·01 N
19 × 19 mm stainless steel	1·44, 1·67, 1·72, 1·37, 1·2
19 × 9·5 mm stainless steel	0·12, 0·20
19 × 6·3 mm stainless steel	0·21, 0·29
30 × 10 mm titanium	1·58, 1·45, 1·1, 1·58
30 × 10 mm small titanium	0·48, 0·5, 0·5

In the old type 500-W Mullard instruments, iodine production was marked (up to 4·5 ml 0·01 M/10 min) but in this instrument as modified by Davies (1959) iodine production was barely detectable (personal communication). As shown recently (Nyborg and Hughes, 1961), free-radical formation by ultrasound is probably dependent on events during the collapse stage of cavitation, which is in turn dependent on pressure-amplitude. Under suitable conditions, some events of cavitation may take place in the absence of collapse and therefore in the absence of free-radical formation (Nyborg and Hughes, 1961).

Cell Disintegration and Iodine Formation by Different Probes

The relation between the rate of free-radical formation, as judged by iodine formation, and cell disintegration was compared with different probes and different volumes of yeast suspension.

Table IX. Iodine formation and yeast disintegration by various probes

Different volumes of the standard KI solution were treated for 10 min and iodine formation estimated as described. The same volume of yeast suspension (1 : 6 w/v) was also treated and the amount of nitrogen released estimated.

Probe	Volume, ml	Iodine, ml 0·01 N	Nitrogen in solution, % total
19 × 19 mm stainless steel	2	0·5	55
	5	1·3	35
	10	1·8	12
	20	1·8	13
	25	1·9	10
19 × 6·3 mm stainless steel	2	0·3	4
	5	0·2	9
	10	0·2	23
	20	0·2	43

In those volumes of cell suspensions where disintegration was approximately the same, the rate of iodine released was markedly different (Table IX). No parallelism has been found between the rate of free-radical formation and the rate of cell disintegration in a wide range of volumes tested. Similar results have been reported by Prudhomme (1957) in experiments in which high-frequency ultrasound (100 kc/s) produced by a quartz crystal was used and sonoluminescence measured as an indication of the chemical effects of ultrasound.

The Inactivation of Alcohol Dehydrogenase and Iodine Formation by Different Probes

Although, as shown in the previous section, there is no parallelism between cell disintegration and the rate of free-radical formation, it seems likely that the inactivation of some enzymes by ultrasound might be directly dependent on free radicals. That this is so in the case of crystalline alcohol dehydrogenase is shown in Table X, where the destruction of enzyme by a probe which caused rapid free-radical formation is compared with the probe causing slow free-radical formation and found to be much greater. That the free radicals are responsible for the rapid inactivation is further supported by the finding that the addition of cysteine or glutathione in amounts equivalent to the iodine formed protected the enzyme against inactivation (Table X). This finding

would account for the high stability, when irradiated by ultrasound, of crude preparations of the enzyme prepared by crushing yeast in the Hughes press (1951), since such preparations contain relatively high amounts of glutathione and other SH-containing compounds. No protection against enzyme inactivation was found by the continuous passage of hydrogen through the solution during treatment.

Table X. The effect of different probes and cysteine on the activity of alcohol dehydrogenase

Ten-ml samples of a solution of crystalline alcohol dehydrogenase were treated for the times stated by two stainless steel probes. Enzyme activity was estimated as described by the method of Racker (1949).

Time, min	Cysteine added, M × 10^3	Enzyme activity, min/ml	
		19 × 19 mm probe	19 × 6·3 mm probe
0	0	8·6	—
2	0	8·4	8·2
5	0	7·0	8·4
15	0	4·6	7·0
10	0	2·2	—
10	2	6·1	—
10	10	6·4	—
10	20	5·6	—
0	0	6·1	—
0	20	5·1	—

It has been previously shown that high molecular weight desoxyribosenucleic acid (DNA) may be rapidly broken down to lower molecular weight fragments by the action of ultrasound. Under the conditions used for these experiments, hyperchromicity was also decreased, probably due to free-radical attack because in more recent experiments where free-radical scavengers have been added, no decrease in hyperchromicity has been found (Doty, McGill and Rice, 1958). A rapid breakdown of calf thymus DNA was found without a reduction in hyperchromicity by treatment with the 30 × 10 mm titanium probe under conditions where free radical formation was slight (Table XI).

Table XI. The effect of ultrasound on Thymus desoxyribosenucleic acid

Twenty-ml samples of DNA (2 mg/ml, 0·05 M NaCl) were treated for the times indicated with a 30 × 10 mm titanium probe. The optical density at 259 mμ was measured immediately and after heating at 100° for 20 min. The molecular weight was determined by light scattering (Peacocke and Preston, 1960). (The author is grateful for permission from Dr. Ian Walker to include these data.)

Time, min	$E_{259\ m\mu}$		Molecular weight $\times 10^6$
	Immediately	After boiling	
0	0·608	15·9	9·0
0·25	0·608	16·0	—
0·5	0·592	15·0	3·3
1	0·592	14·5	1·3
2	0·592	17·0	1·0

Continuous-Flow Treatment

The treatment of cell suspensions by continuous-flow through a sound field is likely to be advantageous, not only because larger volumes could be uniformly treated than in batch methods but also because there would be less inactivation of sensitive material by further comminution and free-radical attack after its release from the cells. Disintegration in a variety of continuous-flow devices attached to the 50-W instrument was too low to be of practical use. From the experience gained, two different shaped vessels have been tested with the larger probe in the 500-W instrument. In the first, the probe was fitted into an inverted T piece with its tip just projecting into the horizontal limb. The rates of disintegration at flow rates of 4–8 ml/min were satisfactory, but due to cavitation unloading, the rate of flow was difficult to control and the temperature of the effluent was often above 30–40° even though maximum power input could not be used (Table XII, Expt. B). More consistent results at higher flow rates were obtained by using highest power input in a vessel in which the suspension was introduced in the bottom and allowed to flow out through a tube placed 2·5 cm above the tip of the probe (Table XII, Expt. A). In both vessels, however, flow rates were lower and temperatures higher than predicted from static experiments. This is possibly due in part to the 'cavitation lag', i.e. the time taken to initiate cavitation in the

Table XII. Continuous-flow treatment of yeast by ultrasound

In Experiment A the large probe was immersed in a 1:6 suspension of yeast so that about 15–20 ml was directly under the end. The yeast suspension was led in from the bottom at the rates given and taken off approximately 2·5 cm above the tip of the probe. The temperature of the yeast on entering was 2°. Each value given is the mean of three separate samples taken for 1 min at 2-min intervals at each flow rate.

In experiment B the conditions were similar to A except that a T-shaped vessel was used in which 12–15 ml was immediately under the large probe.

	Flow rate, ml/min	Power input, scale units	Temperature, °C	Dry wt in super., %
A	0	7	14	30
	10	9·6	20	14·3
	10	10·0	15	14·9
	10	6·6	15	7·0
	5–6	6·7	10	7·3
	8	8·7	19	18·8
	8	10	21	24·0
B	6	7·0	11	15
	7–8	8·0	15	22
	4–5	8·0	22	30
	4–6	8·8	22	39
	4	8·8	35	40

fluid newly entering the sound field (Willard, 1953). Disturbances due to the flow of liquid past the probe may also affect the rate of disintegration.

The Release of Cell Components during the Ultrasonic Treatment of Yeast

In experiments on the effect of atmospheric pressure on cell disintegration by ultrasound, it was found that certain components, such as nucleotides and alcohol dehydrogenase, appeared to leak from the cells very quickly (Neppiras and Hughes, 1962). Similar results have now been found at the early stages (2–5 min) of treatment by the present disintegrators (Fig. 3). Microscopic examination in both the phase-contrast and electron microscope of cells treated for 2–3 min with the 50-W disintegrator showed very few empty or damaged cells even though the release of protein, alcohol dehydrogenase and 260 mμ absorbing material was detected (Fig. 3). Cell counts (Rodgers and Hughes, 1960)

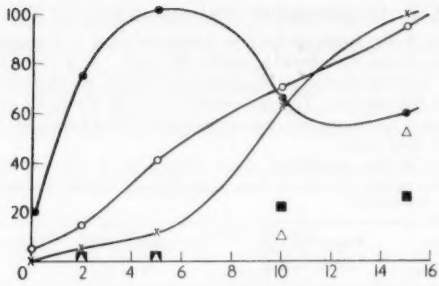

Fig. 3. The release of enzymes and soluble nitrogen at different times of treatment. Thirty ml of a 1 : 6 yeast suspension was treated for the times stated with the 58 : 20 mm titanium probe at power input 5·5. Samples were centrifuged for 10 min at 6,000 × *g* and the N content, alcohol dehydrogenase (Racker, 1949), aconitase, (Green, Mü and Kohout, 1955), fumarase (Massey, 1955), and succinic dehydrogenase measured. Enzyme activities are expressed as units of enzyme/mg N_2/ml.

○—○ N_2 in solution; ●—● alcohol dehydrogenase;

×—× aconitase; ■ fumarase; △ succinic dehydrogenase.

are too imprecise for quantitive measurement during the early stages of treatment. Comparison of total and specific activities of alcohol dehydrogenase show that this enzyme in particular is released early in the treatment, and before any marked cell damage has occurred. Dialysis of the cell extracts suggested that small nucleotides were preferentially released during the early stages of treatment and that there was a further release of 260 mμ absorbing material, probably largely nucleic acids, at later stages. As would be expected, enzymes associated with particulate fractions of the cell (Utter, Nossal and Keech, 1954) are released only when the walls of the majority of cells are ruptured and are beginning to fragment (Fig. 3). Electron micrographs of ultra-thin sections of yeast were too unsatisfactory (cf. Agar and Douglas, 1957) to yield information as to the injury to the cells at stages before fragmentation. At the later stages, although some relatively intact cell walls are found, it is clear that empty cell walls fragment rapidly under these conditions in contrast with some other methods of disintegration (Hughes, 1951; Rodgers and Hughes, 1960).

Table XIII. The disintegration of micro-organisms other than yeast.
(A) 50-W instrument; (B) 500-W instrument

	Organism and enzyme	Conditions	Time, min	Nitrogen in solution, % total
	Lactobacillus arabinosus	20 ml with	5	2
	(18 g wet wt./20 ml)	19 × 6·3 mm probe	30	26
	(Lactic and pyruvate oxidase)	8 ml with	5	36
		19 × 19 mm probe	30	76
A	*Micrococcus aureus*	12 ml with	5	11
	(2g wet wt./12 ml)	19 × 19 mm probe	310	35
	(Hexokinase)			
	Pseudomonas fluorescens	10 ml with	7	50
	(20 g wet wt./20 ml)	19 × 19 mm probe	15	81
	(Nicotinic hydroxylase)			
	Akaligenes faecalis	58 : 20 mm probe	5	70–80[a]
	(4 g wet wt./25 ml)	power 5		
	(Oxidative phosphorylation)			
	Rhodospirillum spheroides	340 ml with	10	[b]
	(95 g wet wt./340 ml)	63 : 32 probe		
	(β-Hydroxybutyrate dehydrogenase)	power 10		
B	*Aspergillus nidulans*	58 : 20 probe	5	60–80[b]
	(4·9 g wet wt./20 ml)	power 6		
	(Hexokinase)			
	Escherichia coli	58 : 20 probe	2	50
	(15 g wet wt./25 ml)	power 6	5	78
	(Ribose *S*-phosphate fermentation)		15	84
	Escherichia coli (B)	63 : 32 probe	6½	[a]
	(125 g wet wt./450 ml)	power 10		
	(Deoxyribonuclease)			
	Haemophilus pertussis	63 : 32 probe	15	80
	(50 g wet wt./400 ml)	power 10		
	(Immunologically active extracts)			
	Pseudomonas ovalis Chester	63 : 32 probe	21	60–80[b]
	(66 g wet wt./250 ml)	power 8·8	48	

[a] Opacity measurements indicated at least 90 per cent of cells destroyed.
[b] Estimated from protein determinations by Folin method.

Table XIV. Comparison of different methods of disintegration of *Corynebacterium xerosis* and the polymetaphosphatase activity of extracts

Cells crushed in the Hughes press contained 14·6 g wet wt./60 ml. Those disintegrated by ultrasound and in the French press contained 11·7 g wet wt./40 ml of which 20 ml was treated by each method. Soluble nitrogen was estimated as described in the text and the value converted to protein by multiplying by 16·2. Polymetaphosphatase was estimated as described by Muhammed, Rogers and Hughes (1960).

Method		Nitrogen in solution		Enzyme activity	
		mg/ml	% total	Units/ml	Units/ml mg protein/ml
Hughes Press	Before	1·82	100	none	
	After	0·80	44	1·62	0·33
Ultrasonic	Before	5·4	100	none	
	After 2 min	0·9	17	0·43	0·08
	After 5 min	2·2	41	0·81	0·06
	After 10 min	2·2	41	0·57	0·04
	After 20 min	4·1	77	0·37	0·02
French Press	1st and 2nd passage	1·24	23	0·11	0·01
	3rd passage	1·86	35	0·42	0·04

Disintegration of Micro-organisms other than Yeast

For the sake of comparison, some results of disintegrating a range of bacteria and micro-organisms are given in Table XIII. These experiments were not designed to test for optimal conditions of disintegration but generally were carried out in order to extract enzymes or other materials as indicated. The results confirm the prediction made that yeast is more difficult to disintegrate than most Gram-negative cells tested, and more easily disintegrated than mycobacteria and Gram-positive cocci and bacilli. The Table also gives a useful illustration of the capabilities of both instruments in a wide range of conditions. In addition, one experiment (Table XIV) is illustrated in which other methods of disintegration (Hughes, 1951; Milner, Lawrence and French, 1950; Rodgers and Hughes, 1960) are compared with ultrasound. The most active preparations of polymetaphosphatase were obtained in this instance by using the Hughes (1951)

press. This enzyme is inactivated by ultrasound but, nevertheless, high activities could be obtained in a series of samples of small volumes of cells (2 ml) much more conveniently and reproducibly than with the Hughes press.

Discussion

The 50-W and 500-W ultrasonic disintegrators described in this paper were designed on the generally accepted assumption that the violent collapse type of cavitation is essential for cell disintegration (Grabar, 1958). A partly immersed velocity transformer (probe), driven by a magnetostrictor as used originally for drilling, was considered likely to give the greatest intensity of cavitation for a given electrical input. This is borne out by the finding that the 50-W generator, can, under certain conditions, cause as much disintegration as the older type 500-W Mullard instrument which operates by driving the bottom of a stainless-steel flask soldered onto the magnetostrictor (Hughes and Rodgers, 1960). Disintegration in stainless-steel vessels was less effective with both new disintegrators and this partly explains the low efficiency of the older type, when much of the available acoustic energy must be lost at the various soldered surfaces and interfaces of liquid, flask and air. The 50-W instrument also compares favourably with the modified 500-W instrument described by Davies (1959) and the newer instrument of Biosonik Ltd. (1961). The new 500-W instrument has a number of advantages over the older and lower powered types, chiefly in flexibility and in the large amounts of suspension disintegrated. Both instruments suffer, however, from the same faults as other sonic methods of disruption in that it is difficult to describe dosage in universally accepted physical parameters which would allow comparison between different designs in use in other laboratories. Some attempt has been made to approach this problem by (*a*) thermometry, (*b*) measurements of amplitude and wave form by means of a strain gauge or more recently in the 50-W instrument and accelerometer (Neppiras and Hughes, 1962), or (*c*) chemical measurements. Although each of the methods has advantages, none is completely satisfactory alone,

but when used together they add considerably to the means of controlling treatment times under defined conditions. Used in conjunction with biochemical studies, these methods of control may also permit a certain degree of predictability in the results to be expected when disintegrating micro-organisms. Nevertheless, it is still impossible to transfer such findings to instruments of different design. This situation will remain until further information concerning the cavitation process and the mechanism by which it disintegrates cells is available. In this connection, and especially with biological material, it is necessary to know how important is the final collapse stage and whether attendant phenomena, such as micro-streaming, which probably takes place before the collapse stage, can cause disintegration (Elder, 1959). The present experiments confirm that cell breakage is not paralleled by free-radical formation, but in view of the effect of free radicals on enzymes and other biological material, it is obviously important to decide whether phenomena which produce free radicals are also necessary for cell breakage.

The role of nucleation in cavitation is still largely unsolved. It is not known whether air or solid or both are needed. The question of the stability of the bubbles which arise from the nuclei is also of importance, especially in constant-flow devices where the time taken to establish cavitation may greatly affect the rate of disintegration. Protein and other substances released from disintegrated micro-organisms are likely, by reason of surface phenomena, to increase the stability of bubbles (Strasberg, 1956). The results of experiments on which powders served as nuclei would support the idea that gas bubbles are more important than solid nuclei.

Surface tension and viscosity can greatly affect cell disintegration. These may alter during the disruption of bacteria and become important. This was marked in the 50-W disintegrator with high-density cell suspensions when the release of high molecular weight DNA and RNA increased the viscosity sufficiently to detune the instrument. Frozen and thawed cells were particularly troublesome. In the 500-W disintegrator the high viscosity often gave rise to cavitation unloading and a rapid local increase in temperature. Constant retuning and adjustment of power input was necessary under these conditions.

The decrease in disintegration caused by CO_2 was most striking and must be taken into account if it is proposed to treat suspensions in growth media where CO_2 may have already been present or may be formed by enzymes released by cell rupture during the early stages of treatment.

Few studies have been made on the over-all effect of ultrasonics on the extraction of various cell components, mainly because the use of these methods is generally directed to the extraction of a single component such as an enzyme or immunologically active fragment. Ultrasonics, as compared with shaking with glass beads, is more effective in solubilizing immunologically active cell fragments; some components are destroyed. The same effects have been found with yeast cells treated in the 50-W disintegrator (Hunt, Rodgers and Hughes, 1959; Hughes and Rodgers, 1960). The study of time constants for the preparation of cell components has also been used to relate enzymology and cell-structure (Marr and Cota Robles, 1957). The present experiments on the time constants for the release of components in the early stages of treatment were carried out in order to gain an insight into the mode of cell rupture. They suggest that, before the complete rupture of the outer cell membranes takes place, certain components may leak from the cells. It is possible that this may be due to disruption of parts of the cell interior brought about by interior streaming motions as described by Nyborg and Dyer (1960). In order to test this idea more fully it is necessary to separate effects due to micro-streaming from those occurring at the collapse stage of cavitation (Nyborg and Hughes, 1961). It is noteworthy that leakage of a similar type through relatively intact cell walls takes place when the cytoplasmic membrane of bacteria is altered by the action of ionic detergents (Hotchkiss, 1946).

It has been found that the best rates of disintegration are obtained when the end of any probe dips from 1.5 mm to 2.0 mm below the surface of the suspension. Particularly, in the 500-W instrument the power level must be adjusted to give maximum noise but no unloading or marked tolerance. The depth of liquid has varied from 0.2 cm to 8 cm in the 50-W and from 1.0 cm to 14 cm in the 500-W instrument. Within these limits the depth of liquid appeared not to markedly affect disintegration. In view

of the reduction of disintegration in narrow vessels it is recommended that a clearance of 0.5 cm is left between the vessel walls and the probe.

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